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TITLE OF THESIS/*TITRE DE LA THÈSE* METABOLITES OF CYATHUS HELENAE AND A RELATED FUNGUS

UNIVERSITY/*UNIVERSITÉ* UNIVERSITY OF ALBERTA (EDMONTON)

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* M.Sc.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

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THE UNIVERSITY OF ALBERTA

METABOLITES OF *CYATHUS HELENAE*
AND A RELATED FUNGUS

by

©

JOHN ROBERT MERCER

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
SPRING 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

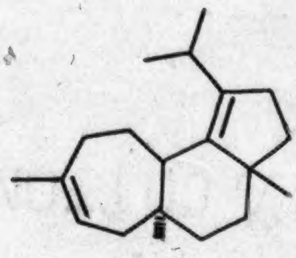
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled METABOLITES OF *CYATHUS HELENAE* AND A RELATED FUNGUS submitted by JOHN ROBERT MERCER in partial fulfilment of the requirements for the degree of Master of Science

W.A.Ayer
W. A. Ayer
Supervisor

Date April 21/75

ABSTRACT

The bird's nest fungus Cyathus helenae has yielded a number of interesting metabolites including a series of diterpenoids with the novel tricyclic skeleton 13[6,7,8,11,12].



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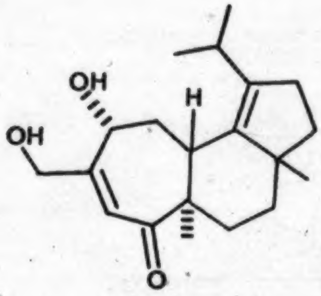
We herein report on the isolation and characterization of neoallocyathin A_4 ($C_{20}H_{30}O_4$), a minor constituent of the crude fungal extract. Proof of the proposed structure 24 was provided by correlation of this compound with cyathin A_3 (3), a compound for which the absolute stereochemistry has been determined by x-ray crystallography.



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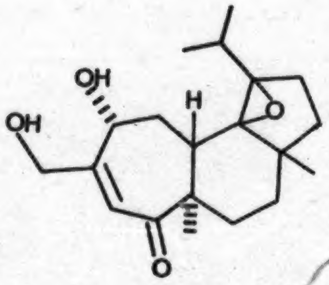

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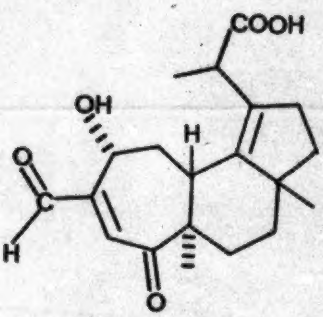
Two other metabolites isolated by earlier workers in this field are discussed. Cyathin C_5 was assigned the tentative structure 37 on the basis of spectral evidence. Preliminary work directed toward the correlation of cyathin A_4, assigned the tentative structure 9, with cyathin A_3, is described.



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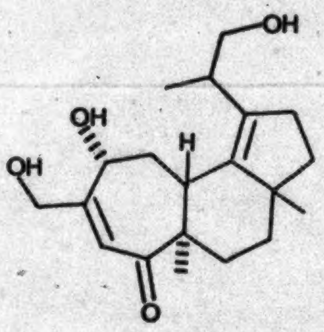

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A preliminary investigation was undertaken on the metabolites of Crucibulum vulgare, another species of birds' nest fungus.

ACKNOWLEDGEMENTS

I wish to thank:

Dr. H. Taube and L. Carstens for an introduction to the research problems and Jo-Ann Forsythe for assistance with the growth of the fungi.

The technical staff of the Department of Chemistry for the determination of the spectra herein.

Dr. David Taylor for proof reading the manuscript and for providing valuable criticisms.

Marcelle Dawe for contributing her expertise to the typing and to the format of the thesis.

My wife, Vida, for encouragement and patience.

Especially, Professor W. A. Ayer, for his supervision of the research and for his many helpful suggestions during all phases of the project.

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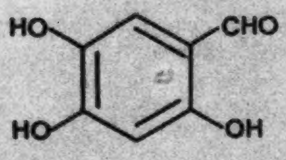
I. INTRODUCTION

In 1966 Brodie reported a new species of bird's nest fungus found growing on dead stems and roots at an altitude of 7000 feet in the Canadian Rockies[1]. The fungus was assigned the class Basidiomycetes, family Nidulariaceae, genus *Cyathus* and was named *Cyathus helenae*.

Olchowecki[2] succeeded in growing *C. helenae* as monosporus mycelia in liquid culture. During work on the sexual characterization of *C. helenae* he noted that a culture of the fungus showed an antagonism to a bacterial contaminant. This chance observation lead to further examination of this antibacterial activity as demonstrated by both *C. helenae* and other closely related species. The antibacterial action was not a novel result with the genus *Cyathus* since antibacterial action had been reported by Broadbent for *Cyathus striatus*[3].

Johri continued the investigation into the antibiotic activity of *C. helenae*[4]. The solid material obtained from evaporation of ethyl acetate extracts of the liquid medium used for cultivation of the fungus was named "cyathin". Cyathin was found to have a broad spectrum of antimicrobial activity against both gram positive and gram negative bacteria and many fungi[4,5]. Johri also carried out nutrition studies and examined the influence of temperature, agitation and aeration on the rate of production of metabolites.

Taube[6] succeeded in separating nine of the components of the crude material. A compound from the crude cyathin with the molecular formula $C_7H_6O_4$ was sparingly soluble in chloroform and was isolated by triturating the total crude with chloroform. The yellow crystalline material named chromocyathin was identified by spectroscopic means and melting point as 2,4,5-trihydroxybenzaldehyde (1). This identification was confirmed by synthesis of authentic 2,4,5-trihydroxybenzaldehyde. Another component of the crude with the same molecular formula ($C_7H_6O_4$) was identified by spectroscopic means as patulin (2).



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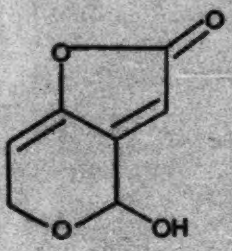

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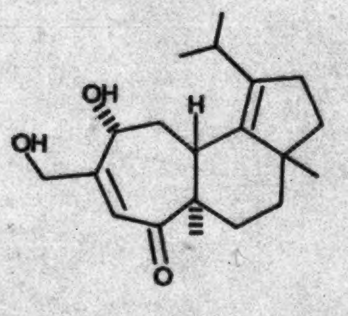
The presence of both these compounds in the crude material lead to speculation on a biosynthetic path which could lead to both compounds from a common intermediate.

Other components of the crude proved to be a series of C_{20} compounds, the members of which appear to have the same basic skeleton. A trivial nomenclature was adopted which named the compounds as "cyathins". The compounds with 30 hydrogens were placed in the "A" series, those with 28 hydrogens in the "B" series and those with 26 hydrogens in the "C" series. A subscript following the series letter denoted the number of oxygens in the molecule. The second compound isolated with the same molecular formula was denoted by the prefix "allo-" and the third compound with the same molecular formula was denoted by the prefix "neoallo-".

The following C_{20} compounds were isolated from crude cyathin: cyathin A_3 ($C_{20}H_{30}O_3$), cyathin A_4 ($C_{20}H_{30}O_4$), allocyathin A_4 ($C_{20}H_{30}O_4$), cyathin B_3 ($C_{20}H_{28}O_3$), allocyathin B_3 ($C_{20}H_{38}O_3$), cyathin B_4 ($C_{20}H_{28}O_4$), cyathin C_5 ($C_{20}H_{26}O_5$). Two of these compounds, cyathin A_3 (3) and allocyathin B_3 (4), were isolated as crystalline materials and their structures were determined by spectroscopic and chemical means[11,12]. The structures were later confirmed by x-ray crystallography of cyathin A_3.



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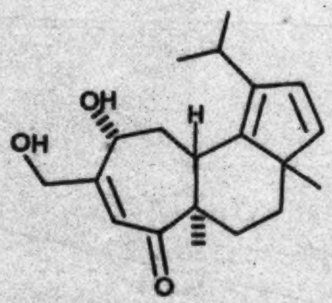

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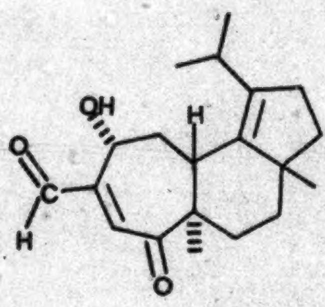
Carstens[7,8] continued the investigation of crude cyathin and isolated three new diterpenoid components. Two of these, cyathin A_2 ($C_{20}H_{30}O_2$) and cyathin B_2 (m/e 300, probable molecular formula $C_{20}H_{28}O_2$), although easily isolated from the crude cyathin defied structural assignment due to their absence from crude extracts taken from later growths of the fungus. The other new diterpenoid isolated was cyathin C_3 ($C_{20}H_{26}O_3$)(6). This compound could not be separated from cyathin B_3 (5) by chromatographic means.

The compounds were eventually separated as their methyl ketals, cyathin B_3 methyl ketal (7) and



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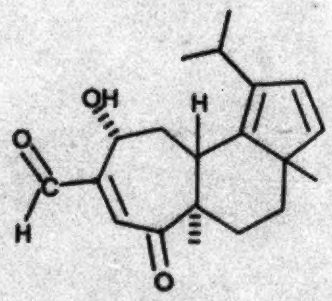

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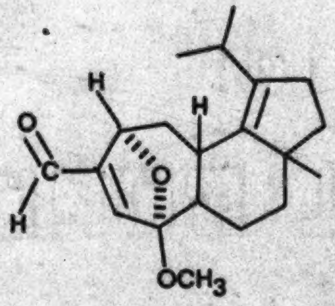

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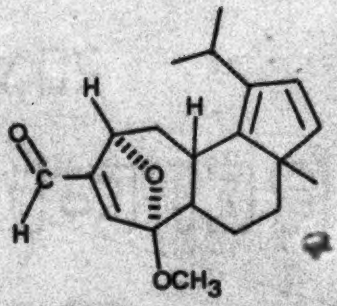

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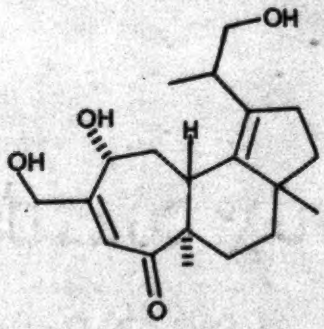
cyathin C_3 methyl ketal (8). Structure determination was carried out on the methyl ketals and confirmation of the assigned structures was obtained by converting the methyl ketals of cyathin B_3 and cyathin C_3 to the methyl ketals of cyathin A_3 and allocyathin B_3 respectively by reduction with sodium borohydride. Carstens also isolated and identified palmitic acid ($C_{16}H_{32}O_2$), a straight chain fatty acid.

Preliminary work was carried out on cyathin A_4 ($C_{20}H_{30}O_4$) by both Carstens[9] and Allbutt[10]. The spectroscopic evidence pointed to structure 9 for cyathin A_4 although this structural assignment has not been confirmed.



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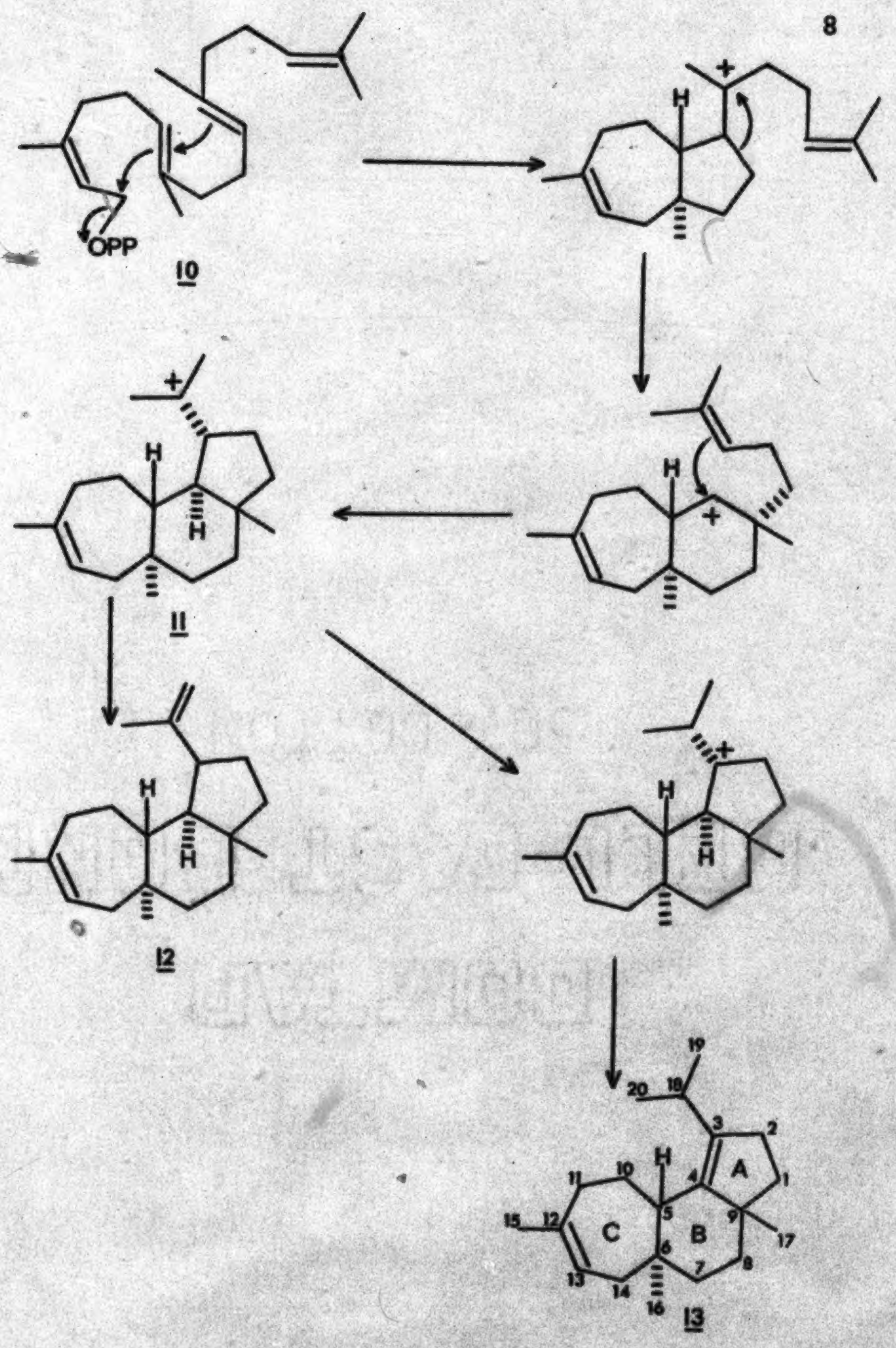
The methods of production and isolation of crude cyathin were developed by Johri[4] and modified by Taube and Carstens. Surface growths of mycelium on liquid culture medium were obtained in several steps from the fruiting bodies of *C. helenae* by standard methods. After a four week growth period the media was extracted with ethyl acetate from which crude cyathin was obtained by evaporating the solvent. The most successful medium for cultivation of *C. helenae* was developed by Brodie

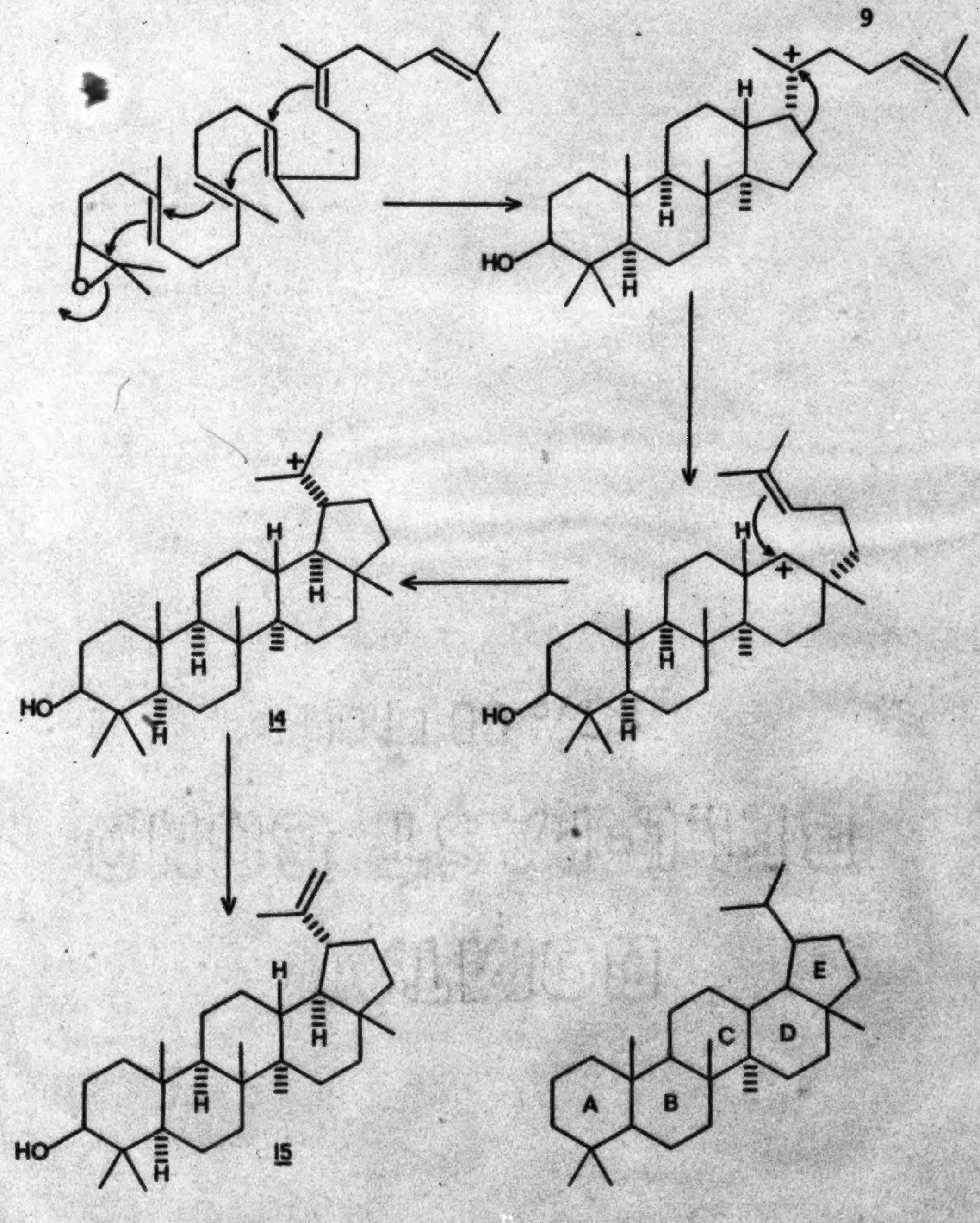
(Brodie medium) for cultivation of another species of bird's nest fungus, Cyathus stercoreus[13].

The biogenesis of the cyathin skeleton has been postulated to proceed as in scheme I. The initial bicyclic diterpenoid formed by cyclization of geranylgeranyl pyrophosphate (10) is transformed to a tricyclic skeleton by a process involving ring expansion and a further cyclization. The ion 11 could lose a proton to form the unsaturated compound 12, but the more favourable process appears to be a hydride shift followed by loss of a proton to give the skeleton 13. No studies have been undertaken to confirm this as the biosynthetic route to the cyathins. However, scheme I does present one plausible approach to the required diterpenoid skeleton. In addition the same basic biosynthetic process is postulated to give the D and E rings of lupeol as in scheme II[14]. After the initial cyclization from squalene oxide the situation is analogous to that in the cyathin molecule. The ion 14 postulated as a precursor to lupeol (15) has identical stereochemistry in the D and E rings as ion 11 has in the A and B rings.

The hydrocarbon 13 itself has not been detected. Individual cyathins may be derived from this skeleton



Scheme I



Lup-20(29)-ene-3β-ol
(Lupeol)

Lupane

Scheme II

through various oxidative and reductive processes occurring at some as yet undefined stage in the biosynthesis.

Other species of bird's nest fungi have been screened for antibiotic activity. Some species which demonstrated both a reasonable antimicrobial activity and a high production of metabolites were examined with respect to the constituent metabolites of the crude extracts. Among the species examined were Cyathus bulleri, Cyathus africanus, Cyathus intermedius and Crucibulum vulgare. At the time of this writing no cyathins have been isolated as constituents of crude extracts from growths of these species. This is not an unexpected result since production of particular fungal metabolites is known to be species or even strain specific.

One of the initial objectives of the work undertaken in this thesis was to prove the structure of cyathin A_4 (9) and to examine other of the more polar constituents in the crude cyathin mixture.

II. RESULTS AND DISCUSSION

Chromatographic Refinement of Cyathin

The crude cyathin obtained from ethyl acetate extractions was generally a brown foam with a distinct musty odor and a complex composition as determined by analytical thin-layer chromatography (tlc). During the course of this work the author cultivated 25 growths of C. helenae on Brodie medium. The quantities of crude material varied from 150 to 600 mg per liter and the production of particular metabolites was by no means regular or predictable.

Fungi are known to be extremely variable organisms and one outward sign of this is the phenomenon of "sectoring"[2,15]. A mutation occurs at the advancing edge of mycelium on a solid support. The result is a wedge or slice of mycelium that has different physical characteristics and/or different biochemical capabilities from the remaining mycelium. The frequency of mutation on solid support indicates that the same process is at work in liquid media. This process may in part account for the variability of metabolites extracted from the media. In several cases metabolites present in early growths disappeared entirely from later growths. This was the case with cyathin A_2 and cyathin B_2 as mentioned previously and also with two other metabolites, cyathin A_4 and cyathin C_5, which will be discussed later in this thesis.

Column chromatography and particularly preparative and analytical tlc have proved invaluable in the separation of cyathin components. Silica gel and silicic acid from various sources were the adsorbents used exclusively in this work. The choice of silica gel and silicic acid was based on the demonstrated utility of these adsorbents in most separations encountered with cyathins. There was a certain prejudice against alumina and polyamide as adsorbents since it was known that they had a pronounced although undefined effect on samples of crude cyathin[6]. Several trials involving the partially purified cyathin components indicated that with these compounds silica gel was superior in separating power and resolution to alumina or polyamide.

Initial tlc was useful in assessing the approximate amounts of various components in crude cyathin. The developed tlc plate was visualized by heating after spraying with sulfuric acid. This procedure indicated the presence of the various components by the position (R_f value) of the spots on the plate. The relative concentration of the components could be approximated from the size of the spots or their degree of charring. A plate treated in this manner is reproduced in figure 1. The eight separations shown are samples of crude cyathin obtained at various stages during this research. All crude "cyathins" were derived from strain 1500 of C. helenae, all were grown on Brodie medium and all were extracted in the same manner. It is obvious that the production of particular metabolites was variable.

Non-destructive location of components after tlc was possible if the silica was impregnated with an inorganic phosphor during preparation of the plates. Addition of 3 to 6% phosphor caused the silica to give a greenish glow when irradiated with short wave ultraviolet (uv) light at 254 nm. Against this bright background the components of the crude appeared as dark spots or areas. These areas invariably corresponded in size and intensity to the result obtained after spraying with sulfuric acid and heating. Familiarity with the usual tlc pattern allowed assignment of various areas in the crude materials to particular

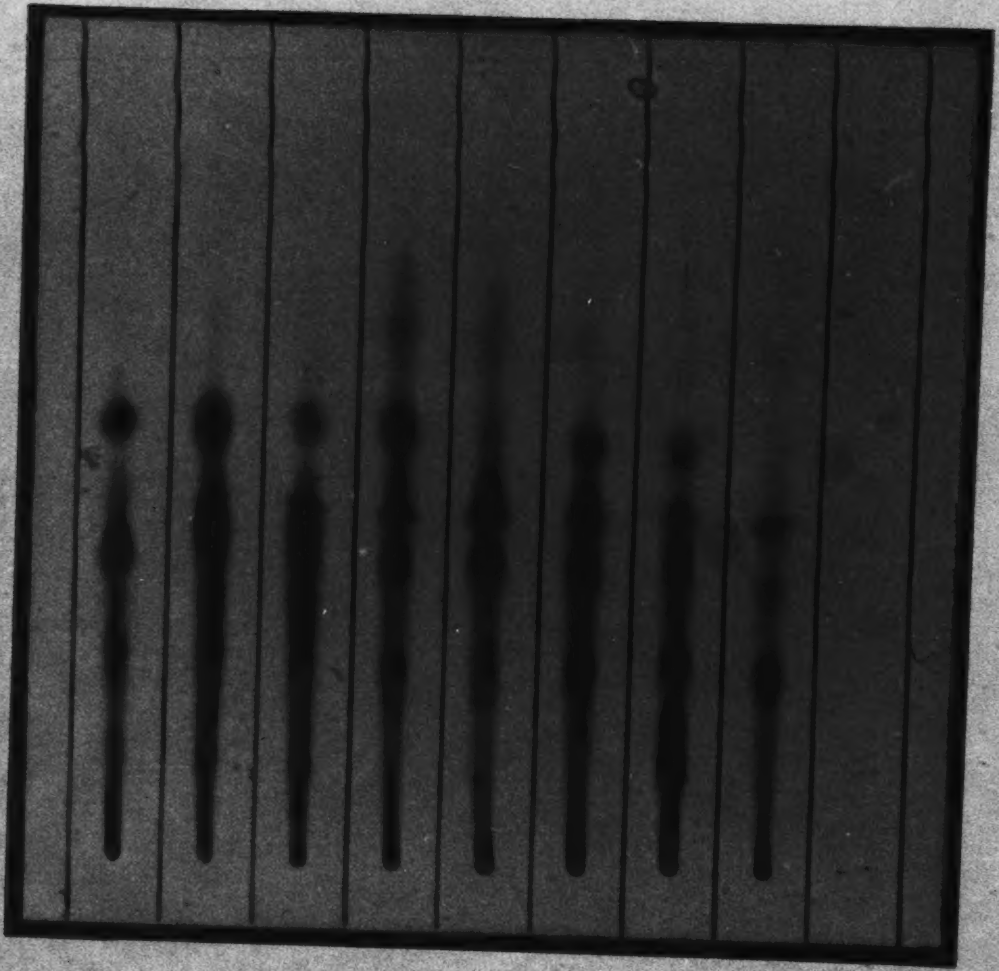


Figure 1. Developed analytical tlc plate containing various samples of crude cyathin; visualized with sulfuric acid spray.

compounds. In cases where there was some doubt as to the identity of a particular component, the crude material was run on the same plate as a sample the composition of which was known, or with samples of pure compounds obtained from previous separations.

Quantities of crude material in the 2 to 3 g range were separated by column chromatography. Silicic acid (Mallinkrodt, 100 mesh) was used with an adsorbent to sample ratio of approximately 50 to 1. Separations were most successful when the initial eluting solvent was distilled reagent grade chloroform (0.75% ethanol stabilizer). More polar components of the mixture were removed from the column by the addition of 1 to 5% methanol to the chloroform.

The progress of the separation could be monitored by ultraviolet light if 1% inorganic phosphor was added to the silicic acid. This procedure required the use of a quartz column which is transparent to uv light of the frequency employed. The presence of compounds was indicated by dark bands visible during irradiation. On other occasions the composition of the eluate was determined by tlc using 25 x 75 mm microscope slides coated with silica gel.

The first material to be eluted during column chromatography was the cyathin B_3 and cyathin C_3 mixture described earlier[7]. This material was obtained in high purity as indicated by a single spot on tlc and several examples of spontaneous crystallization of column fractions upon removal of solvent (cyathin B_3 (5) and cyathin C_3 (6) form mixed crystals of variable composition). Other constituents of the crude extract were not obtained in such high purity. This was particularly true of the more polar compounds.

Further purification of column fractions was carried out by preparative thin-layer chromatography (ptlc). The improved separations with ptlc were in part due to the higher adsorbent to sample ratios used, typically 250:1 or better, and in part due to refinements in plate preparation and sample application (see detailed experimental). In the latter stages of this work ptlc was used on crude extracts without preliminary refinement by column chromatography.

Isolation and Proposed Structure of Neoallocyathin A_4

1. Mixture containing neoallocyathin A_4

Cyathin A_4 with the proposed structure 9 was reported as the main component of the more polar fractions from column chromatography[9,10]. Mass spectra of several fractions from a particular column separation seemed to indicate the presence of this compound by the appearance of an apparent parent ion at m/e 334. This is the expected molecular ion for cyathin A_4 ($C_{20}H_{30}O_4$). Ptlc on the column fractions yielded a material which was a single spot on tlc and which retained m/e 334 as a prominent ion.

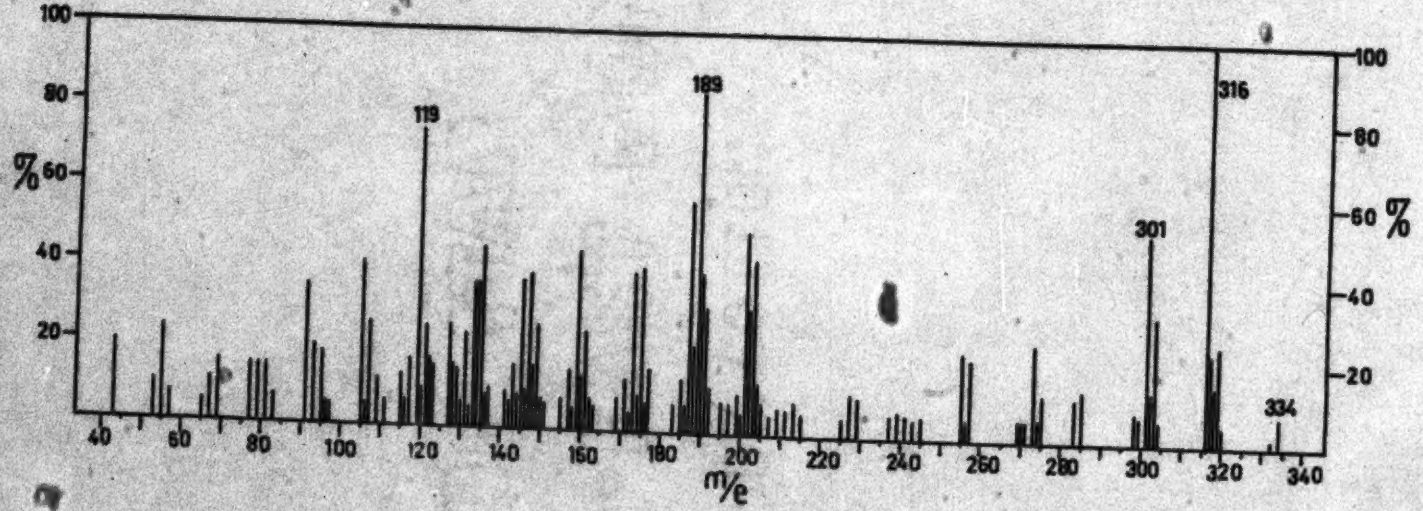
However, direct comparison with authentic cyathin A_4 indicated that the material isolated was a new component of crude "cyathin" not previously reported. Not only were the mass spectra considerably different but the two materials showed different R_f values on tlc [cyathin A_4: R_f (A)0.07, R_f (E)0.56; new m/e 334 compound: R_f (A)0.32, R_f (E)0.73 (see detailed experimental for a description of solvent systems and calculation of R_f)].

The new compound was assigned a probable molecular formula of $C_{20}H_{30}O_4$ and the nomenclature system discussed previously dictates the name neoallocyathin A_4 for this compound.

It soon became obvious that the fraction containing neoallocyathin A_4 was a mixture of several components. The main evidence for the complexity of this material came from the mass spectrum (figure 2). The spectrum contains several peaks not readily derived from the parent ion at m/e 334, notably m/e 318 and 332.

The use of silver nitrate impregnated silica gel (argentated silica gel) was examined as a method of further separation of this mixture. This adsorbent had been used successfully for the separation of cyathin A_3 (3) from allocyathin B_3 (4)[6] and also for the separation of cyathin B_3 methyl ketal (7) from cyathin C_3 methyl ketal (8)[7,8]. The formation of π complexes between olefinic unsaturations in the organic molecule and silver ions on the silica surface causes the more unsaturated compounds to have a greater affinity for this adsorbent. De Vries[16] had utilized this adsorbent for the separation of cholesterol from cholestanol. The adsorbent is also known to show some degree of selectivity with double bonds

Figure 2. Mass spectrum of mixture containing neoallocyathin A_4.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301
316
334
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140
160
180
200
220
240
260
280
300
320
340
m/e

Figure 2

relative to their geometry in the molecule and to their degree of substitution.

When the mixture containing neoallocyathin A_4 was subjected to ptlc on argentated silica gel, using freshly prepared plates, two well defined bands were located: R_f (E)0.59 and R_f (E)0.50. The bands were removed from the silica and the compounds isolated from the adsorbent and subjected to mass spectral analysis.

The material at low R_f proved to be almost entirely allocyathin B_3 (4). The mass spectrum (figure 3) compares favourably with previously published spectra for this compound (figure 4). The R_f values on ordinary and argentated silica gel were identical with authentic allocyathin B_3 in several solvent systems. Later characterization of this low R_f component as its acetonide and O,O-diacetyl derivatives confirmed its identity (see detailed experimental). The mass spectrum also reveals the presence of a compound with m/e 332. This material was assumed to be a minor component, not only because of the small size of the molecular ion but also because of the lack of any notable fragments from m/e 332 in the spectrum.

Figure 3. Mass spectrum of low R_f component from argentated silica gel separation of the mixture containing neoallocyathin A_4.

Figure 4. Mass spectrum of allocyathin B_3.

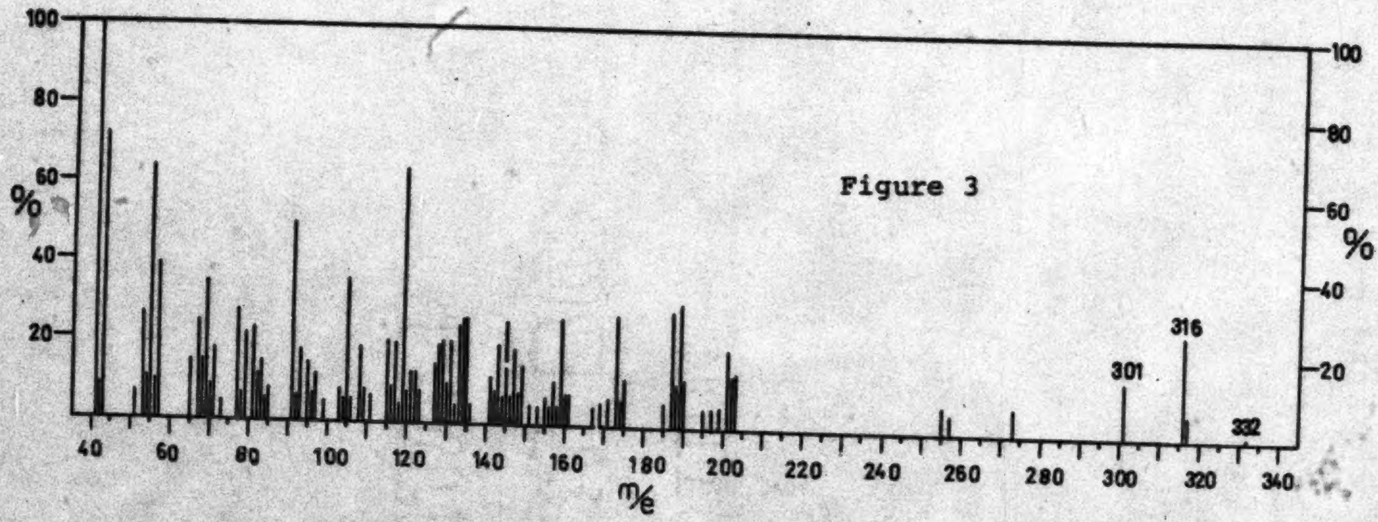


Figure 3

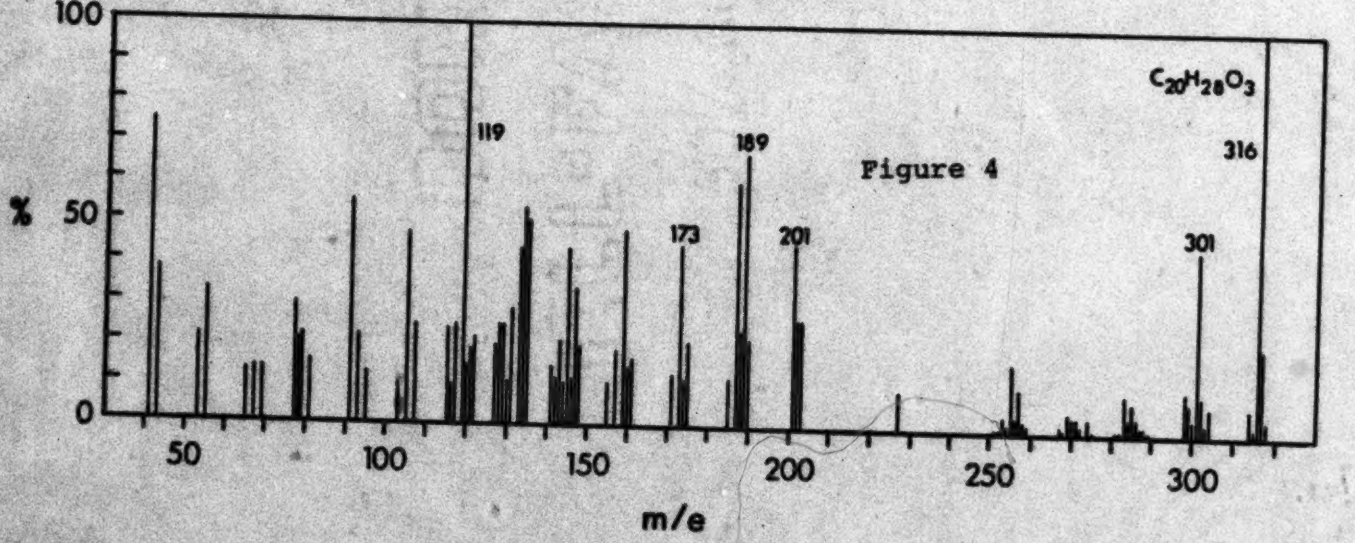


Figure 4

The material at high R_f contained mainly cyathin A_3 (3). The mass spectrum (figure 5) compares favourably with a mass spectrum of this compound published previously (figure 6). The R_f value of the isolated material was identical on ordinary and argentated silica gel with that of an authentic sample of cyathin A_3. In this case, however, the presence of a considerable amount of a second compound, namely neoallocyathin A_4, is evident from the mass spectrum.

High resolution mass spectrometry (hrms) varified the tentative molecular formula of $C_{20}H_{30}O_4$ for neoallocyathin A_4.

2. Isolation of neoallocyathin A_4 as its acetonide derivative.

On acetylation cyathin A_3 readily forms a diacetate, the spectral details of which have been reported earlier (reference 6, page 46). It was hoped that after acetylation the O,O-diacetylcyathin A_3 could be separated by chromatography from the acetylation product of neoallocyathin A_4.

Treatment of the mixture of cyathin A_3 and

Figure 5. Mass spectrum of low R_f component from argentated silica gel separation of the mixture containing neoallocyathin A_4.

Figure 6. Mass spectrum of cyathin A_3.

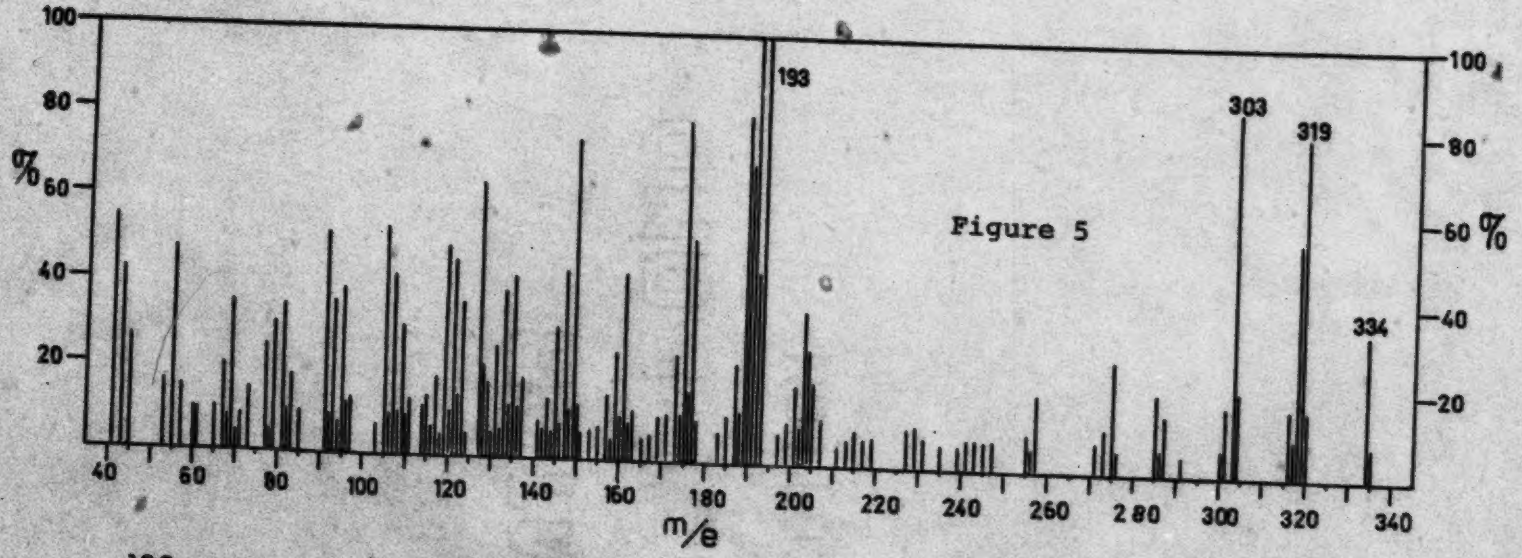


Figure 5

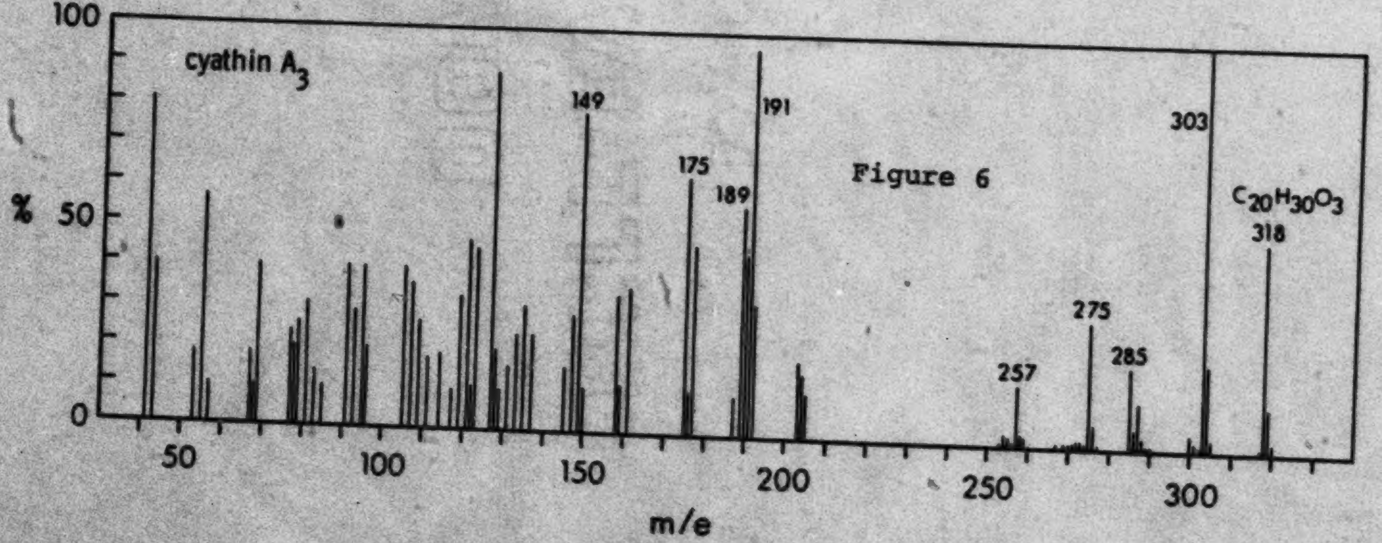


Figure 6

neoallocyathin A_4 with pyridine and acetic anhydride in methylene chloride gave one major product which was isolated by ptlc: R_f (A)0.65. Several minor products noted on the preparative plates were not isolated. In the ir spectrum (figure 7) and the mass spectrum (figure 8) the indication is that both cyathin A_3 and neoallocyathin A_4 have formed diacetates. Figure 8 shows m/e 402 for O,O-diacetylcyathin A_3 and m/e 418 for a diacetate of the neoallocyathin A_4. The infrared spectrum is quite similar to that for cyathin A_3 diacetate and indicates that there are no hydroxyl groups remaining on either component of the mixture.

The 100 MHz nuclear magnetic resonance (nmr) spectrum determined on the mixture has a signal at δ 6.11 for an olefinic proton vicinal to a carbonyl, signals at δ 5.35 and 4.65 for a methylene group vicinal to a primary acetoxyl and a methine proton vicinal to a secondary acetoxyl group. There are also two sharp singlets at δ 2.01 and 2.09 for the acetyl methyl groups. All these signals were indicative of O,O-diacetylcyathin A_3 although the complexity of the spectrum, particularly in the C-methyl region, clearly indicates a mixture of components.

Figure 7. Infrared spectrum ($CHCl_3$) of acetate mixture, mainly O,O-diacetylcyathin A_3.

Figure 8. Mass spectrum of acetate mixture, mainly O,O-diacetylcyathin A_3.

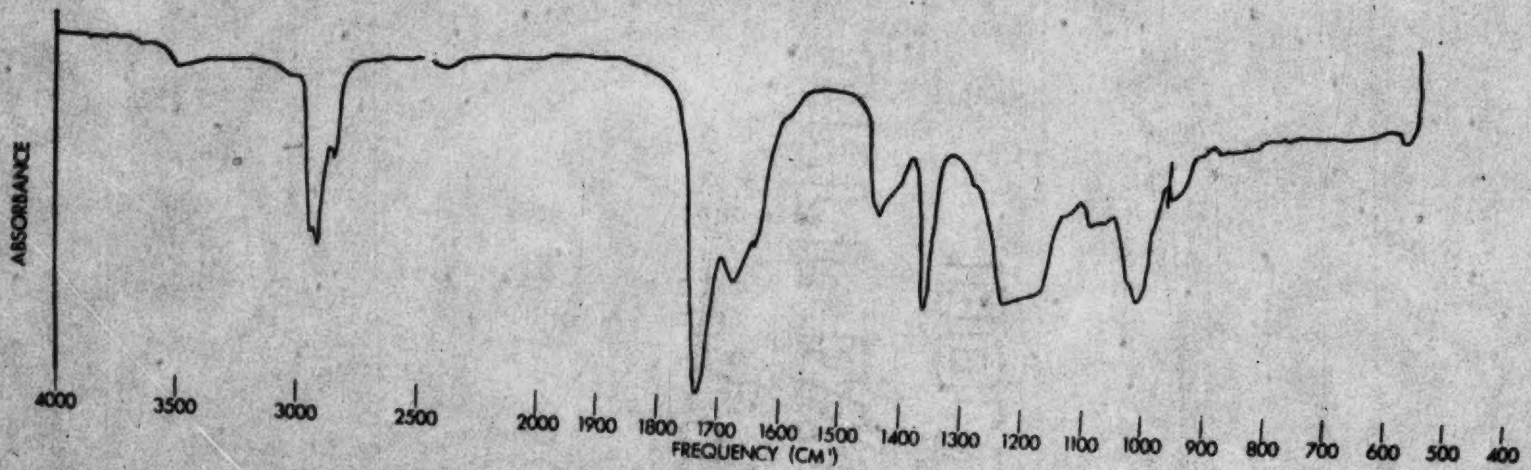


Figure 7

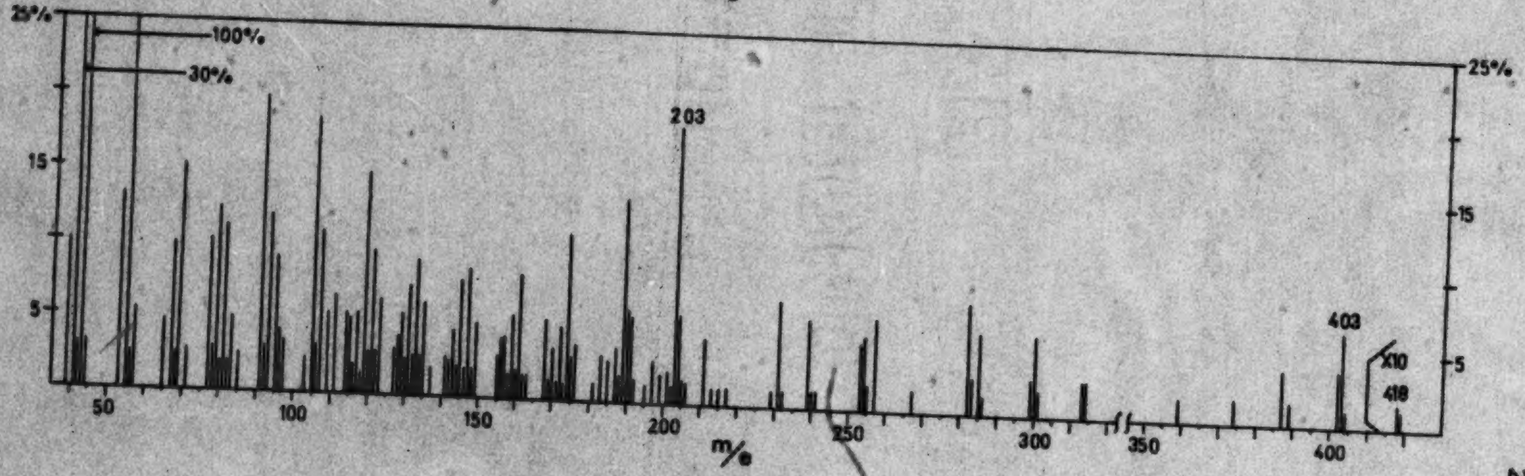


Figure 8

Attempts to separate the mixture of acetates by tlc using various solvent systems and employing ordinary and argentated silica gel were unsuccessful.

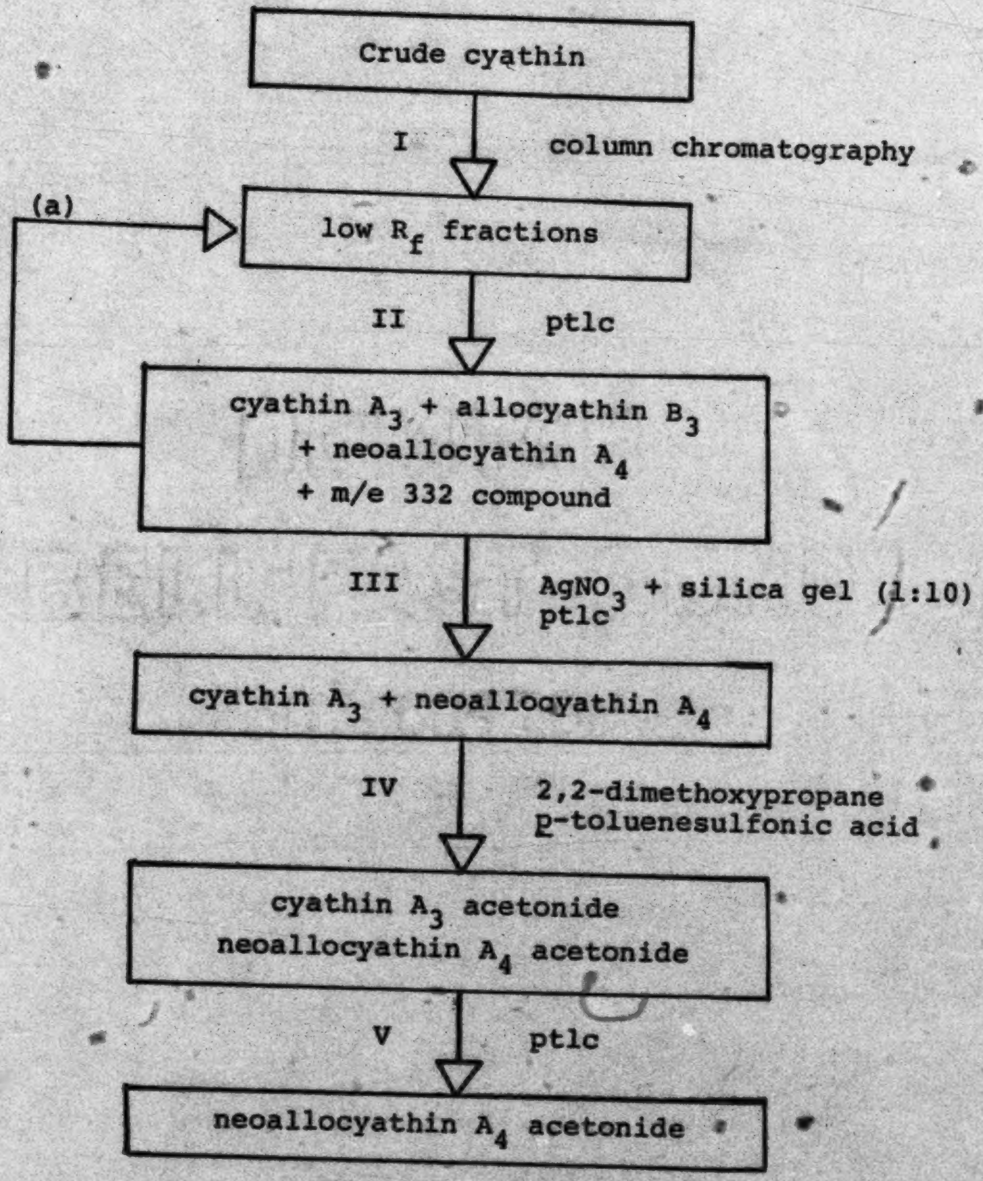
Suitably disposed 1,2- and 1,3-diols are known to react under various conditions to form isopropylidene derivatives (acetonides). This reaction had been employed extensively to block the two hydroxyl groups in the C ring of cyathin A_4[10]. Acetonide formation was considered as a method of separation of cyathin A_3, containing a suitably disposed diol, from neoallocyathin A_4 which might not contain the required structural feature for derivative formation.

Acetonides have been prepared by various methods such as reacting the diol with acetone and a mineral acid or acetone and an anhydrous salt. The most useful method in this case was found to involve solution of the diol in 2,2-dimethoxypropane followed by addition of a catalytic amount of p-toluenesulfonic acid to catalyze ketal exchange.

Treatment of the mixture of cyathin A_3 and neoallocyathin A_4 in this manner gave a mixture of acetonides. This indicated that both starting materials had hydroxyl groups suitably disposed for acetonide formation.

Several attempts at separation of the acetonides were carried out. Solvent system B was found to give rise to two spots on tlc. The lower spot, R_f (B)0.49, burned violet after spraying with sulfuric acid and heating while the upper spot, R_f (B)0.52 burned brown. The higher R_f spot proved to be a mixture of acetonides while the mass spectrum of the lower R_f component indicated that it was a single compound. Further trials with ptlc separation gave clean separation of these two materials. The high R_f compound was found to be cyathin A_3 acetonide by comparison with authentic material synthesised from crystalline cyathin A_3 (5). The lower R_f material was neoallocyathin A_4 acetonide (as indicated by the mass spectrum) containing only traces of impurities.

The isolation of neoallocyathin A_4 as its acetonide derivative requires five steps as outlined in scheme III. The first isolation of the derivative yielded 2 mg of material from 1.01 g of crude cyathin, or approximately a 0.2% yield. Scheme IV outlines several of the separations carried out on crude cyathin to yield neoallocyathin A_4 acetonide. The new compound was never isolated in more than 0.25% yield and in several cases it could not be detected in the crude. The low percentage of neoallocyathin A_4 in the crude mixture was the main problem in further characterization of the material. The combined



(a) repeated ptlc was employed at this stage in several separations

Scheme III

WEIGHT OF CRUDE CYATHIN	1.01 g	2.2 g	1.18 g(b)	.627 g(b)	.800 g(b)
Weight of product after purification step (mg) Yield of product after purification step (%)					
I(a)	214.9 21.2	835 38.0	- -	- -	- -
II	56.8 26.6	144.3 17.5	108.3 9.2	89.2 14.2	101 12.6
III	14.9 26.2	51.4 35.6	25.9 23.9	36.1 40.3	41 40.6
IV	8.8 59.0	22.4 43.8	- -	- -	- -
V	2.0 22.7	2.6 11.6	0 0	1.1 3.2	2.0 4.9
Overall yield (%)	0.2	0.12	0	0.18	0.25
Overall yield (mg)	2.0	2.6	0	1.1	2.0

(a) numbered steps refer to the purification steps pictured in scheme III

(b) column chromatography was omitted in these separations and the products from step III were treated by ptlc without the isolation of reaction products

Scheme IV

weight of natural neoallocyathin A_4 isolated during this work was less than 10 mg. Spectra show that this material still contained traces of impurities that had not been removed in the isolation steps.

Some decomposition of samples was evident. Initially purified samples gave multiple spots on tlc and the character of certain spectra varied if the samples involved had been allowed to stand at room temperature or in solution.

3. Proposed structure of neoallocyathin A_4.

The mass spectrum of neoallocyathin A_4 acetonide (figure 9) shows a parent peak at m/e 374. Exact mass on this peak gave the molecular formula $C_{23}H_{34}O_4$. This is the expected formula for addition of an isopropylidene group to a diol of molecular formula $C_{20}H_{30}O_4$ and hence is a confirmation of the molecular formula of the naturally occurring alcohol. The molecular ion at m/e 374 loses a methyl radical to give m/e 359. No other prominent peaks are noted in the high mass range.

An ir spectrum on the isolated acetonide gives absorptions characteristic of cyathins (figure 10). The

Figure 9. Mass spectrum of neoallocyathin A_4.

Figure 10. Infrared spectrum ($CHCl_3$) of neoallocyathin A_4 acetonide.

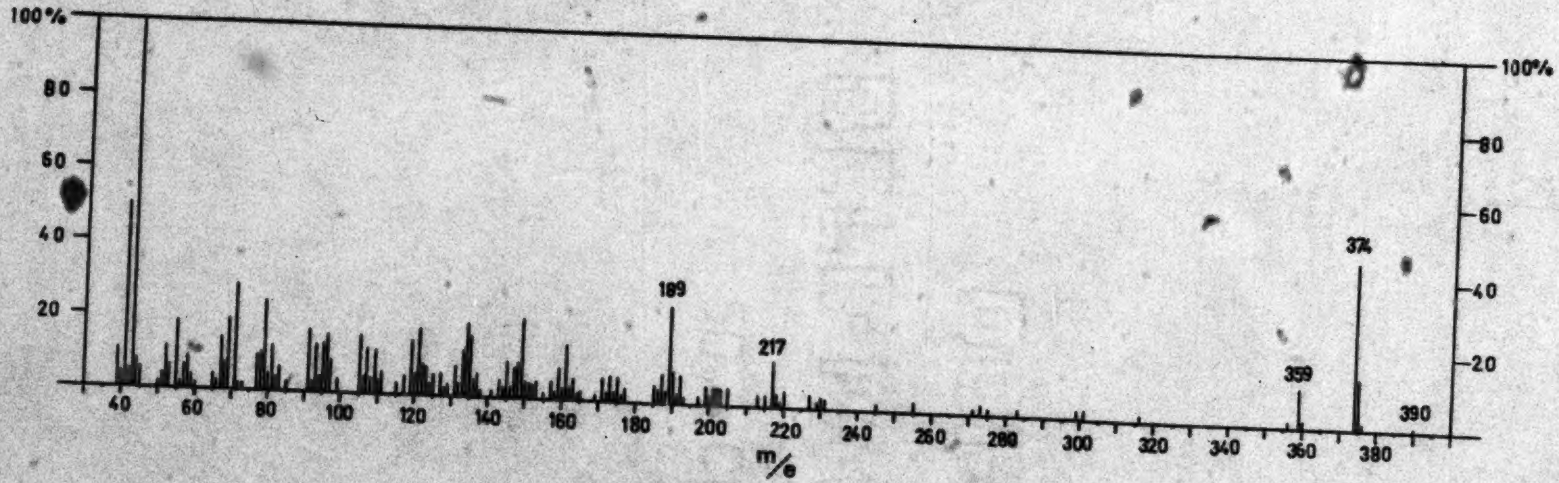


Figure 9

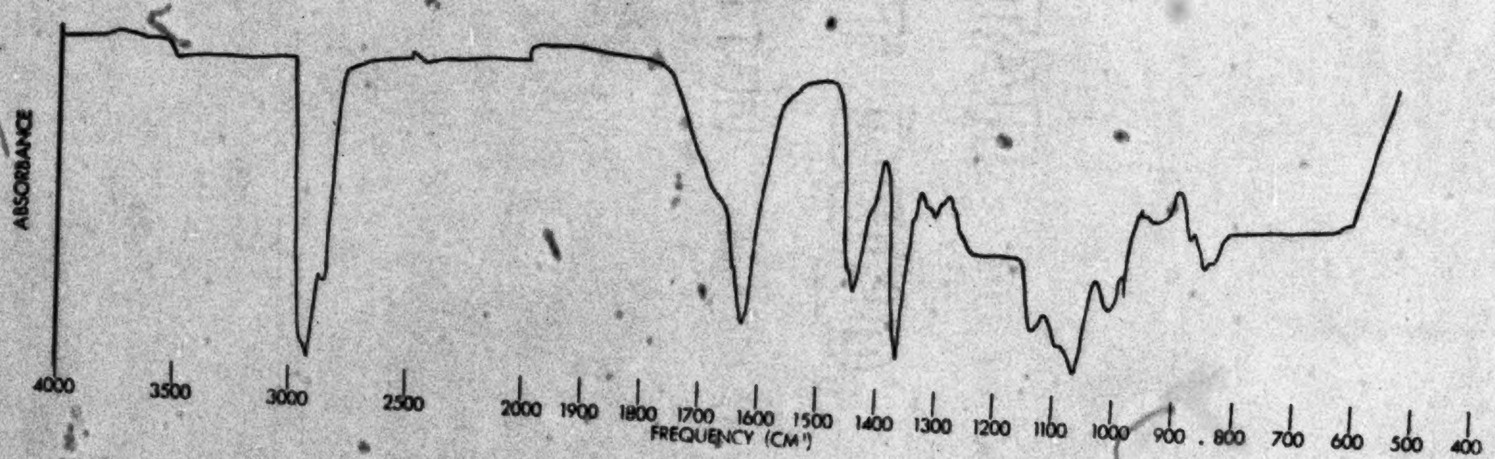


Figure 10

absence of hydroxyl groups was evident in the ir spectrum of the mixture of diacetates (figure 7). The lack of absorptions above 3000 cm^{-1} in figure 10 confirms this earlier evidence. The band at 1635 cm^{-1} is similar to the band for the α,β-unsaturated ketone found in the C ring of other cyathins.

The nmr spectrum reproduced in figure 11 is the best spectrum obtained thus far on the acetonide of the natural product. This spectrum was determined using the proton Fourier transform (HFT) method on a 100 MHz nmr instrument. This method allows excellent spectra to be determined on samples smaller than 2 mg. Other samples of the acetonide derivative of the natural product showed the same signals as in figure 11 but in addition displayed signals due to various impurities which made these spectra very difficult to interpret.

The sharp signal at δ 7.25 is attributed to traces of $CHCl_3$ in the $CDCl_3$ used as a solvent. The signal at δ 5.74 can be attributed to a single olefinic proton. Its position is comparable to that of the olefinic proton in the system

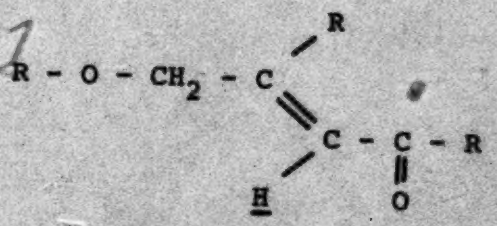


Figure 11. Nuclear magnetic resonance spectrum ($CDCl_3$) of neoallocyathin A_4 acetonide.

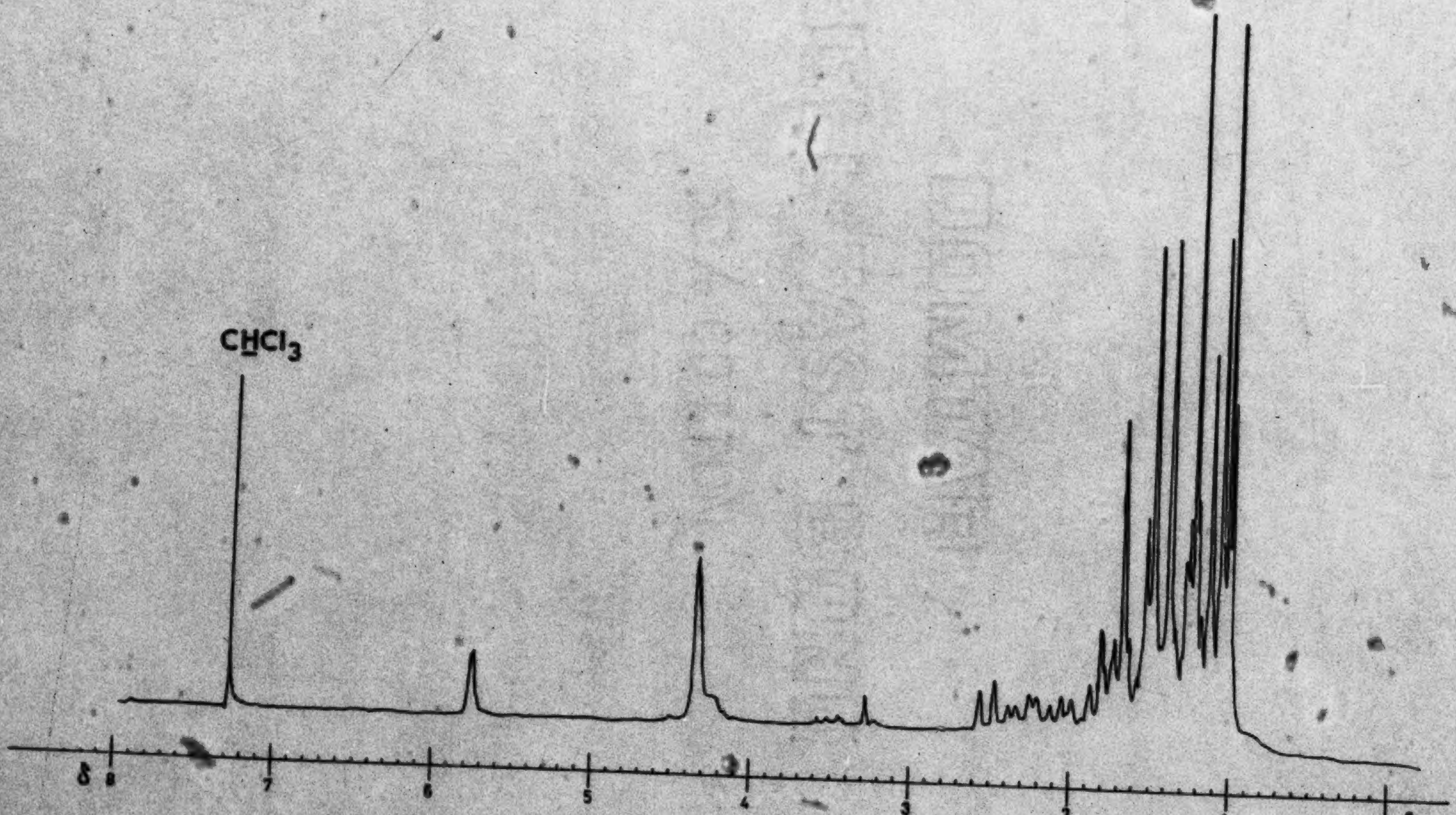


Figure 11

which occurs in other cyathins.

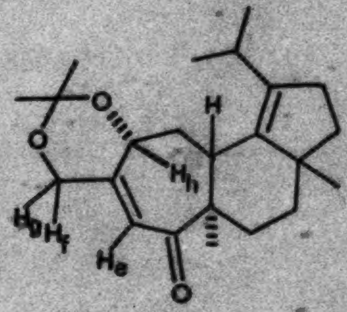
Integration can not be obtained for HFT spectra and the area under the signals was estimated by tracing around the peaks and comparing the weights of the tracings. Two signals appear to coincide in the region δ 4.15 to 4.35. A somewhat broadened two proton singlet at δ 4.34 partially obscures a more diffuse signal in the region from δ 4.15 to 4.35; the presence of which is also evident from the shoulder on the high field side of the signal at δ 4.34.

Two singlets at δ 1.50 and 1.40 are due to the acetonide methyl groups. The slightly downfield position of these methyls is attributed to the β-relationship to the ethereal oxygens. Two other sharp signals occur at δ 1.24 and 1.03 indicating two isolated methyl groups. The remaining signals in this area appear as an apparent triplet centered at δ 1.08. The central signal of the triplet is somewhat broadened indicating that the apparent triplet is in fact two overlapping doublets centered at δ 1.11 and 1.04. An attempt to resolve this signal by expansion of the spectrum was not successful and a spectrum determined in C_6D_6 did not simplify the signals in this region.

The combined chemical and spectral data described above allowed the tentative assignment of a structure to neoallocyathin A_4.

The molecular formula $C_{20}H_{30}O_4$ indicates six sites of unsaturation, three of which are accommodated in rings if we assume the tricyclic cyathin skeleton. Neoallocyathin A_4 must contain two of its oxygens as hydroxyl groups since a diacetate is formed upon acetylation and the hydroxyl groups must have a 1,2- or 1,3-relationship to account for the facile formation of the acetonide. A third oxygen occurs as an α,β-unsaturated ketone as indicated by the ir.

The nmr was particularly valuable in assigning a structure for neoallocyathin A_4, particularly when the spectrum was compared with that of cyathin A_3 acetonide (16) (figure 12).



16

Figure 12. Nuclear magnetic resonance spectrum ($CDCl_3$) of cyathin A_3 acetonide.

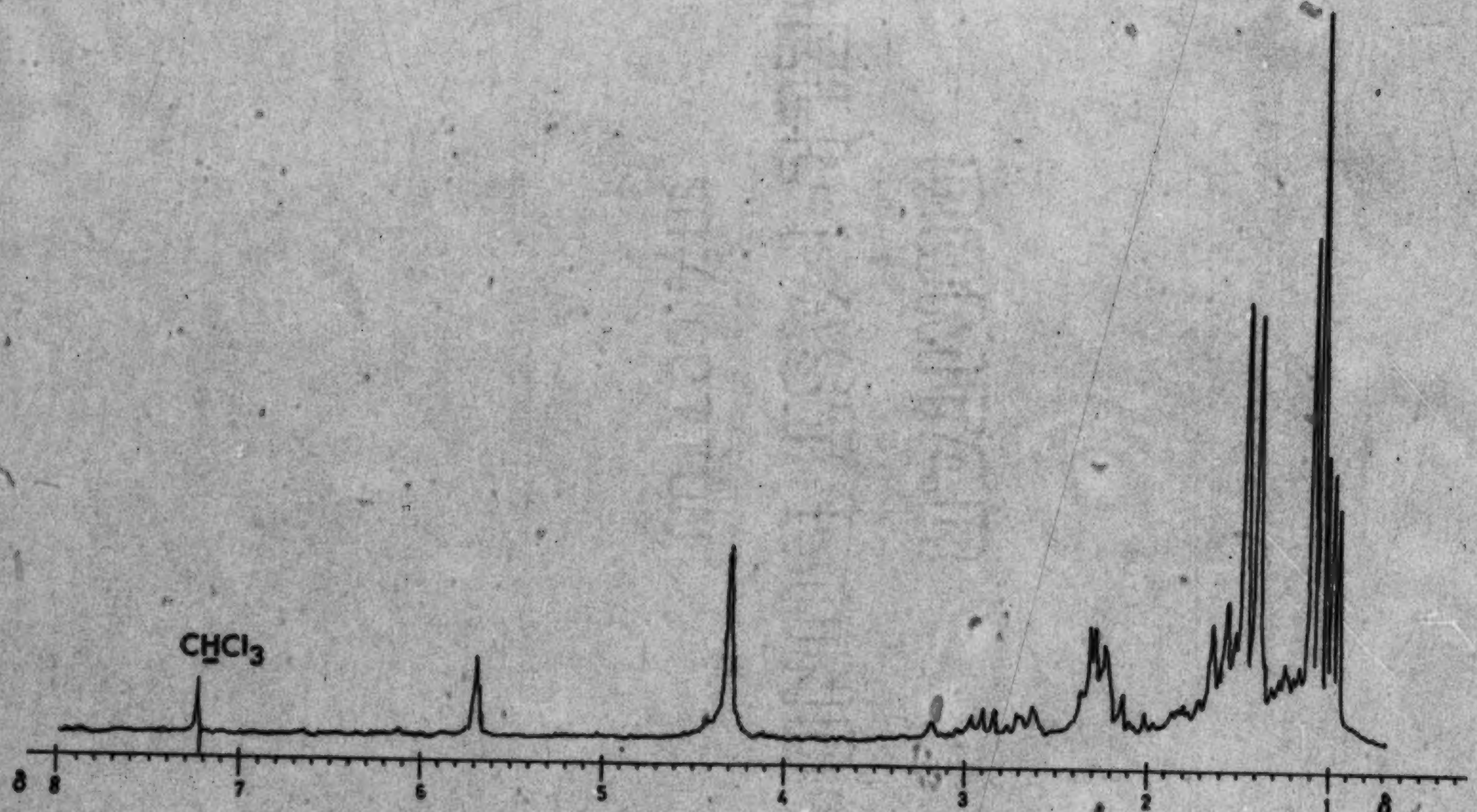


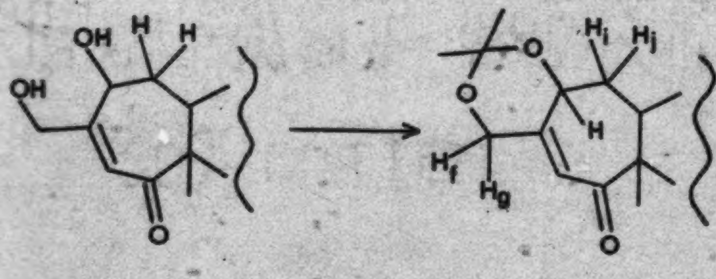
Figure 12

Cyathin A_3 acetonide was obtained during the final step in the chromatographic isolation of neoallocyathin A_4 acetonide. Other samples were prepared by treating crystalline cyathin A_3 (3) with 2,2-dimethoxypropane and p-toluenesulfonic acid. This is the first reported preparation of the acetonide derivative of cyathin A_3 although several other derivatives have been prepared[6].

The nmr spectra of the two acetonides (figures 11 and 12) suggest a close structural relationship between neoallocyathin A_4 and cyathin A_3 (3). The signal for proton e in cyathin A_3 acetonide occurs as a singlet as does the signal for protons f and g. Both these singlets have some fine splitting due to allylic coupling. Signals similar to these two occur in the spectrum of the unknown compound (figure 11). The chemical shift of the signals for proton e and for protons f and g occur at δ 5.68 and 4.29 respectively while the comparable signals in neoallocyathin A_4 occur at δ 5.74 and 4.34.

The two spectra also contain a signal partially obscured by the signal due to the protons f and g. In cyathin A_3 acetonide this is assigned to proton h which is expected to occur as a doublet of doublets at this position.

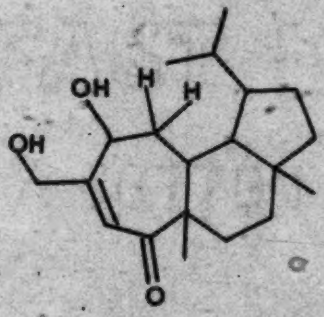
The close similarity of the low field portions of the two spectra suggests that neoallocyathin A_4 acetonide contains the partial structure 18 comparable to cyathin A_3 acetonide and hence structure 17 may be postulated as a partial structure of the starting diol.



17 18

The nmr spectra suggests further similarities between the two compounds. In cyathin A_3 acetonide the methyl region contains two singlets for the acetonide methyls, two singlets for the angular methyl groups and two doublets assigned to the isopropyl methyl groups. The non-equivalence of the isopropyl methyl groups is presumably due to restricted rotation around the C-3 and C-18 bond or to their asymmetric environment. This feature has been observed in other cyathins containing this structural feature. The spectrum for neoallocyathin A_4 acetonide contains signals comparable to those in the methyl region of cyathin A_3 acetonide.

This evidence suggests that neoallocyathin A_4 contains the intact skeleton of the cyathins and the partial structure can now be written as 19.

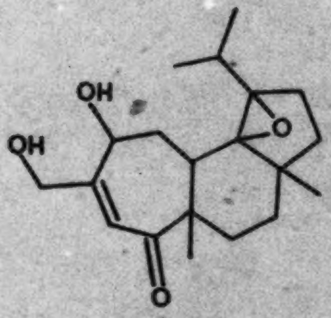


19

Structure 19 has the molecular formula $C_{20}H_{32}O_3$. The correct molecular formula for neoallocyathin A_4 is achieved by adding one oxygen and one site of unsaturation to structure 19.

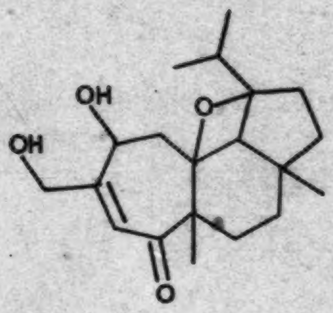
The failure to observe the remaining oxygen by ir suggests that it exists as an ether and the required double bond equivalent can be accommodated by constructing a cyclic ether. The carbons in the C-O-C linkage must be tertiary to explain the failure to observe a signal for the system H-C-O-R in the nmr spectrum, other than those for protons f, g and h as discussed previously. The carbons C-3, C-4, C-5 and C-18 satisfy this requirement. C-18 is excluded on the basis of the nmr which requires a proton at this position to split the methyl signals for C-19 and C-20.

The cyathins identified by previous workers were uniform in containing an unsaturation in the A ring between C-3 and C-4 and this suggests structure 20 as the most likely candidate for neoallocyathin A_4.



20

Although all spectral details discussed to this point are consistent with 20 they do not rule out alternate cyclic ethers 21 and 22.



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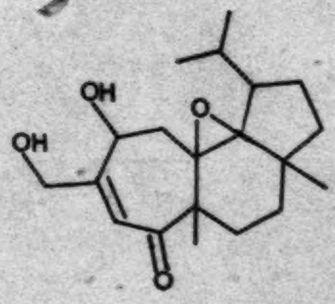
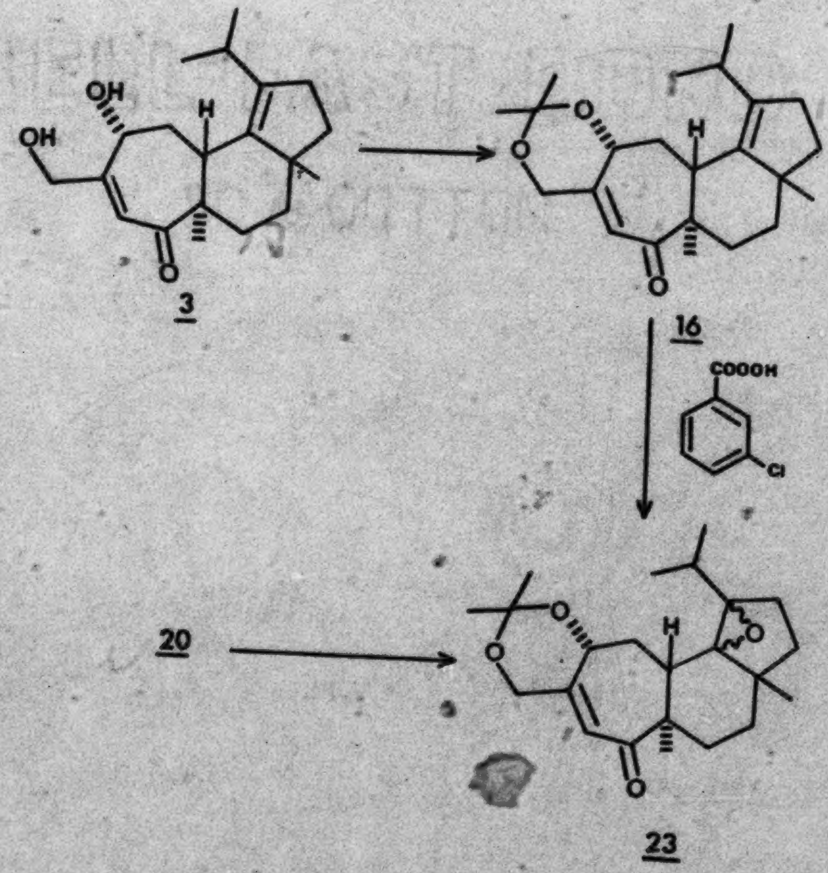


22

Correlation of Neoallocyathin A_4 with Cyathin A_3

The close structural relationship between cyathin A_3 (3) and the proposed structure for neoallocyathin A_4 (20) suggests a correlation between the two as shown in scheme V. It was hoped that synthesis of the epoxide 23 would permit assignment of both the structure and stereochemistry of neoallocyathin A_4.



Scheme V

Several samples of cyathin A_3 acetonide were combined and purified by ptlc. The purified sample was dissolved in benzene and treated with approximately one equivalent of m-chloroperbenzoic acid. The tlc behaviour and mass spectrum of the reaction product indicated that epoxidation had taken place and that the product was similar to the natural material. The nmr spectrum gave conflicting evidence. All the signals present in the spectrum of the natural acetonide were present in the spectrum of the semi-synthetic material but these signals were accompanied by additional signals which appeared as shoulders on the high field side of the expected signals.

Several explanations were considered for these discrepancies in the nmr spectrum. The extra peaks could be due to unreacted starting material. Alternately, an excess of epoxidizing agent may have lead to epoxidation of the second double bond in the molecule. Normally, α,β-unsaturated ketones are resistant to epoxidation under the conditions used. However, the presence of a peak at m/e 390 in the mass spectrum, which is the correct mass for the bis-epoxide, leaves this possibility open. A third proposal is that both diastereoisomeric epoxides had been formed. This appears unlikely since models of cyathin A_3 acetonide indicate that the β-face is less hindered than the α-face. The main steric effect on the α-face would arise from the C-6 methyl and the axial protons at C-8 and C-10 (β-face refers to the front face of the molecule as drawn while α-face indicates the back face of the molecule).

A sample of cyathin A_3 (3) from material crystallized by H. Taube was converted to the epoxide by the two steps shown in scheme V. It was interesting to note that a small sample of neoallocyathin A_4 acetonide was separated as an impurity in the samples of cyathin A_3 (3) crystallized by Taube. This second epoxidation was carried out in chloroform with care taken to ensure that precisely one equivalent of peracid was used. The product was pure by tlc, R_f (B) 0.49, and the mass spectrum (figure 13) and nmr spectrum (figure 14) were almost identical with the corresponding spectra of the natural product acetonide (figures 9 and 11). Both the natural and semi-synthetic materials contain traces of impurities since the nmr of both compounds contain small signals not duplicated in the other.

Since cyathin A_3 (3) of known structure and stereochemistry has been unambiguously converted into an epoxide, the structure of which is identical with the natural product, we have confirmed our original assignment of the natural product (20).

Figure 13. Mass spectrum of epoxidation product from cyathin A_3 acetonide.

Figure 14. Nuclear magnetic resonance spectrum ($CDCl_3$) of epoxidation product from cyathin A_3 acetonide.

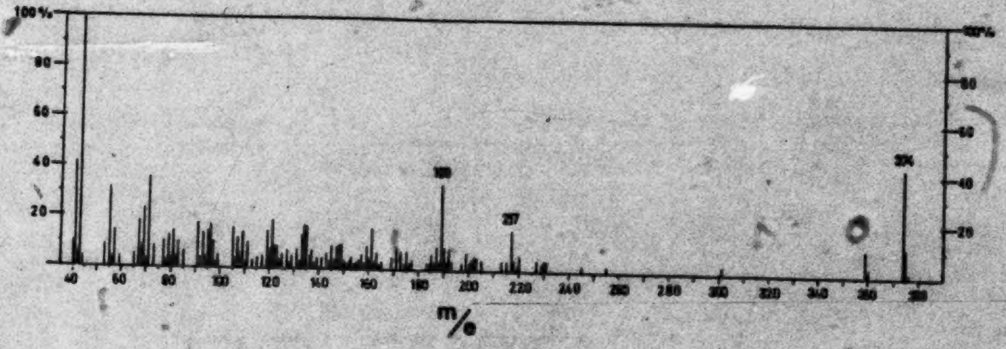


Figure 13

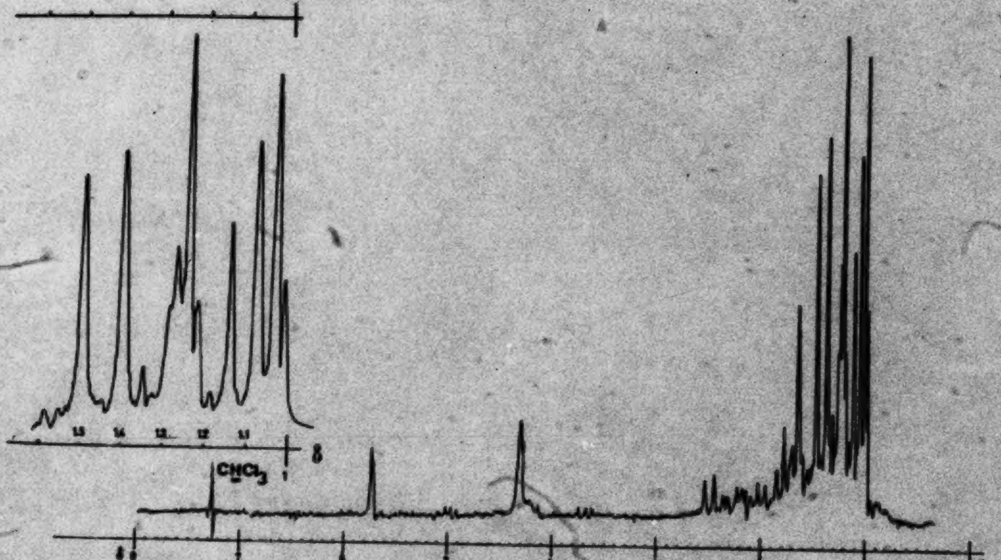
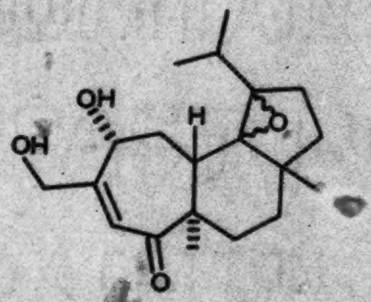


Figure 14

The relative stereochemistry of optically active centers would not be altered by the reaction sequence described in scheme V and this allows assignment of the relative stereochemistry of all centers in the molecule except the terminal positions of the epoxide. Structure 24 therefore represents the structure and stereochemistry of the natural product neoallocyathin A_4.

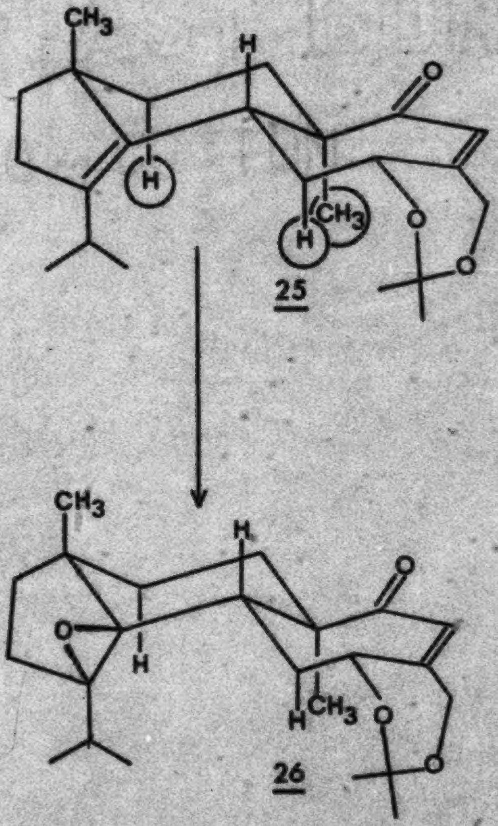


24

Circular dichroism (cd) and optical rotatory dispersion (ord) spectra have been determined on the natural and synthetic materials and will be discussed in detail later. The close resemblance of the chiroptical spectra obtained from the two materials confirms that the absolute stereochemistry of the natural and synthetic materials is the same. An x-ray analysis of cyathin A_3 (3)

was used to confirm its relative and absolute stereochemistry as depicted in structure 3.

As mentioned earlier, molecular models suggest that the β-face of cyathin A_3 acetonide (25) is less hindered than the α-face (especially with respect to 1,3-diaxial interactions) and although there is no direct evidence available, it is tentatively suggested that neoallocyathin A_4 acetonide is the β-epoxide 26.



25

26

Additional Data Relevant to the Structure of Neoallocyathin A_4

1. Nmr data

Analysis of the nmr spectrum of neoallocyathin A_4 acetonide (26) (figure 11) was aided by comparison with the spectrum of cyathin A_3 acetonide (10) (figure 12) as discussed previously. These two spectra were examined further along with the spectrum of allocyathin B_3 acetonide (27) (figure 15). This examination was expected to lead to a differentiation between the C-16 and C-17 angular methyl protons by the change in chemical shift of the latter when the structural features in the A ring were varied. In addition, it was hoped that chemical shift variations between neoallocyathin A_4 acetonide and the related acetonides would give additional evidence in support of the proposed β-configuration for the epoxide ring.

The data from the nmr spectra are reproduced in tables 1, 2 and 3. The proton assignments are given for neoallocyathin A_4 acetonide in figure 16.

The nmr chemical shifts of protons i and k in neoallocyathin A_4 acetonide (table 1) were assigned after a spin-spin decoupling experiment. Irradiation of proton

Figure 15. Nuclear magnetic resonance spectrum ($CDCl_3$) of allocyathin B_3 acetonide.

Figure 16. Proton assignments for neoallocyathin A_4 acetonide.

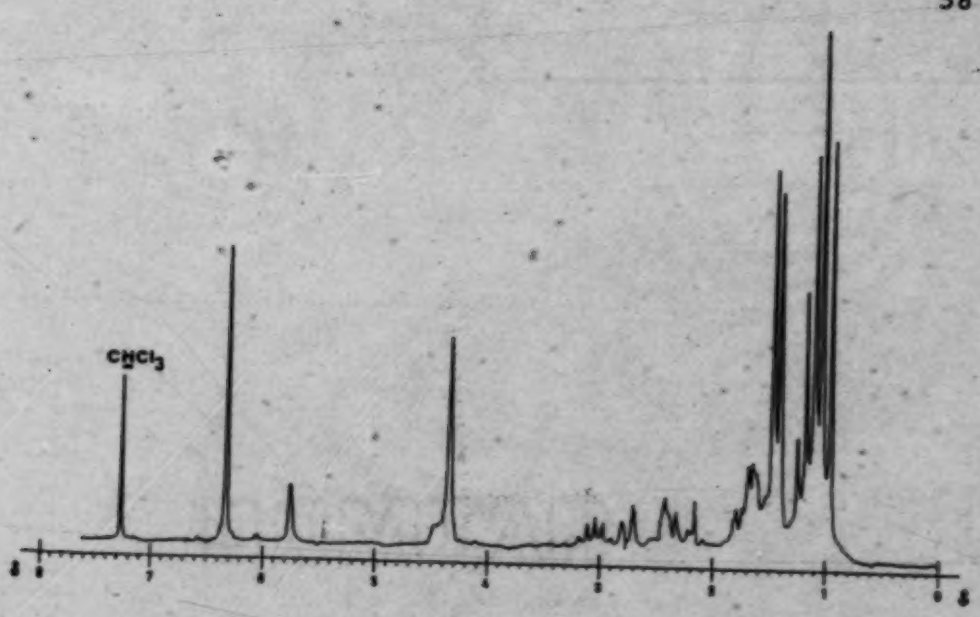
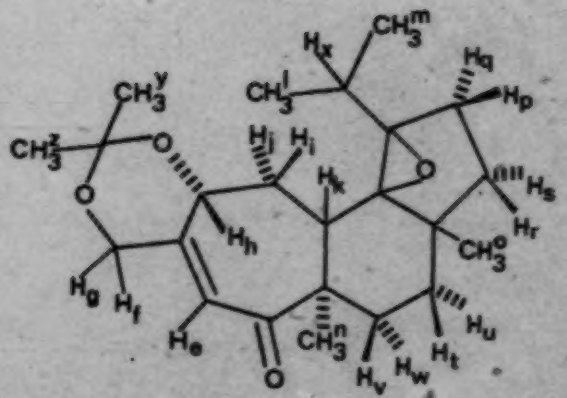


Figure 15



For allocyathin B_3 acetonide: Hq + Hp = Hc and Hr + Hs = Hd

Figure 16

Nmr data for neoallocyathin A_4 acetonide (20)

Signal	Shift in $CDCl_3$ (δ)	Multiplicity	Coupled with signals	Coupling constant (Hz)
e	5.74	m	f,g	small (allylic)
f,g	4.34	m	g,f,e,	small
h	~4.15* (?)	dd	i,j	4,?
i	2.30	ddd	j,k,h	13,0,4
j	?	ddd	i,k,h	13,9,?
k	2.50	dd	i,j	0,9
l (m)	1.11	d	x	6
m (l)	1.04	d	x	6
o (n)	1.24	s	-	-
n (o)	1.03	s	-	-
p,q,r,s, t,u,v,w	2.15-1.20	?	?	?
x	?	m	l,m	6,6
y (z)	1.50	s	-	-
z (y)	1.40	s	-	-

* Underlined data is postulated on the basis of the position of the proton in the molecule although the multiplicity and coupling constant may not be visible from the spectra

Table 1

Nmr data for cyathin A_3 acetonide (10)

Signal	Shift in $CDCl_3$ (δ)	Multiplicity	Coupled with signals	Coupling constant (Hz)
e	5.68	m	f,g	small
f,g,	4.29	m	g,f,e	small
h	~4.35* (?)	dd (?)	i,j	?
i,j	2.50-2.00 (?)	?	j,i,h,k	?
k	2.68	dd	j,i	9,?
l (m)	1.02	d	x	6.5
m (l)	0.99	d	x	6.5
o (n)	1.10	s	–	–
n (o)	1.06	s	–	–
p,q,r,s, t,u,v,w	2.00-1.1	?	–	–
x	2.92	m	?	?
y (z)	1.47	s	l,m	6.5,6.5
z (y)	1.40	s	–	–

* Underlined data is postulated on the basis of the position of the proton in the molecule although the multiplicity and coupling constant may not be visible from the spectra.

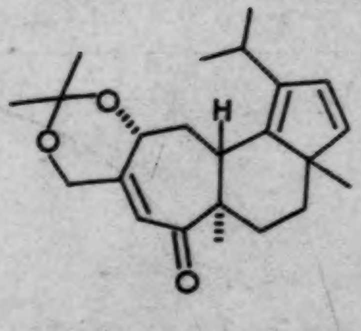
Table 2

Nmr data for allocyathin B_3 acetonide (21)

Signal	Shift in $CDCl_3$ (δ)	Multiplicity	Coupled with signals	Coupling constant (Hz)
c,d	6.29	s	-	-
e	5.72	m	f,g	small
f,g	4.30	s	-	-
h	4.30-4.45* (?)	dd (?)	i,j	?
i,j	2.60-2.00 (?)	?	j,i,h,k	?
k	2.77	dd	j,i	9,2
l (m)	1.16	d	x	6.5
m (l)	1.09	d	x	6.5
n (o)	1.07	s	-	-
o (n)	0.98	s	-	-
t,u,v,w	1.90-1.20	?	?	?
x	3.06	m	l,m	6.5,6.5
y (z)	1.49	s	-	-
z (y)	1.44	s	-	-

* Underlined data is postulated on the basis of the position of the proton in the molecule although the multiplicity and coupling constant may not be visible from the spectra.

Table 3



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h at δ 4.15 led to the collapse of the doublet of doublets centered at δ 2.30 to a doublet at the same position with J=13Hz. The signal at δ 2.30 must therefore be due to either proton i or j. The absence of coupling between proton k and the proton at δ 2.30 can be explained if the dihedral angle between proton k and either proton i or j is approximately 90°. In a model of the epoxide, attempting to move proton j into the required relationship with proton k leads to distortion of the seven membered ring to the point where the C-C double bond and the carbonyl are no longer in the same place. At the same time the acetonide ring is forced into the crowded α-face of the molecule. Neither of these unfavourable conditions arise when proton i is moved into the required relationship with proton k.

It seems likely, therefore, that proton i gives rise to the signal at δ 2.30.

Decoupling of proton h did not reveal the signal from proton j to which it is presumably also coupled. This signal and the signal for proton x are obscured in the complex region below δ 2.15.

The preceding tables show the following data for the angular methyl signals:

cyathin A_3 acetonide	δ 1.10, 1.06
allocyathin B_3 acetonide	δ 1.07, 0.98
neoallocyathin A_4 acetonide	δ 1.24, 1.03.

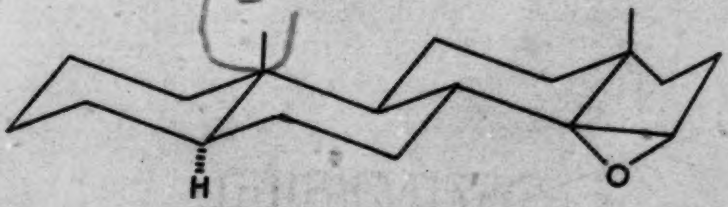
Variations in the A ring of the molecules are expected to have a more pronounced effect on the C-17 methyl protons (o protons) while the more remote C-16 methyl protons (n protons) should be only slightly influenced. In going from cyathin A_3 acetonide (10) to allocyathin B_3 acetonide (27) the signal at δ 1.06 in the former compound probably corresponds to that at δ 1.07 in the latter. These signals can then be tentatively assigned to the n protons.

The upfield shift of the o protons can be accounted for by the extra diamagnetic anisotropy associated with the cyclopentadiene ring. This would be expected to shield the o protons which lie above the plane of this five membered ring.

In neoallocyathin A_4 acetonide the signal at δ 1.03 should arise from the n protons, this being only slightly shifted from the values of δ 1.06 and 1.07 for the other two acetonides. The signal at δ 1.24 must therefore arise from the o protons. The low field position of this signal would be due to either the removal of the shielding influence of the double bond present in cyathin A_3 acetonide or a deshielding effect associated with the epoxide ring.

The significant downfield shift of the angular methyl protons on C-17 was examined in an attempt to relate this shift to the configuration of the epoxide ring in the molecule. In steroids, the nmr signals due to the angular methyls (C-18 and C-19) are known to vary in a regular way depending on the substituents on various parts of the molecule. Substantial tables of additivity relationships were complied by Zurcher[17,18] and summarized elsewhere[19].

Two compounds, 14α, 15α-oxido-5α-androstane (28) and 14β, 15β-oxido-5α-androstane (29) are relevant to the present discussion since the stereochemical situation in the C and D rings of the steroids are comparable to the situation in the two possible epoxides, the partial structures of which are shown in 30 and 31.



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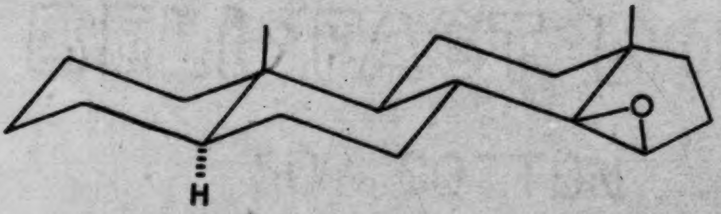

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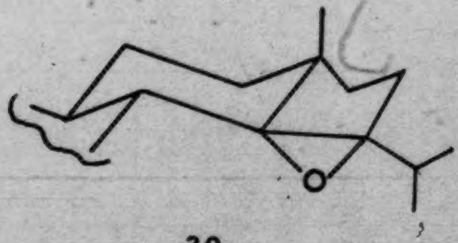

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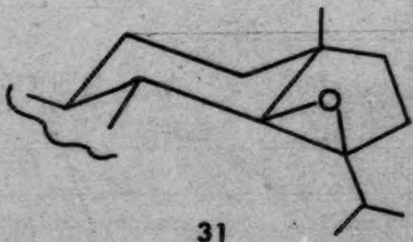

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Unfortunately, the additivity values for the 14,15-epoxy steroids are similar for α- or β-orientation. For the α-epoxide 28 the additivity value is 0.183 ppm, this value being added to the value for the C-18 protons in the hydrocarbon. For the β-epoxide 29 this additivity factor becomes 0.150[19]. The small difference in these values is not sufficient to distinguish between the α- and β-epoxides of neoallocyathin A_4 acetonide, particularly since the relationship relevant to the steroid case would only apply in a general sense to the cyathin molecule.

It was also considered possible that the epoxide ring could affect either proton i or proton j in a manner that would indicate the configuration of the epoxide ring. The epoxide ring is known to shield protons lying above the plane of the ring and this shielding has been attributed to ring currents[20].

Examination of the nmr tables presented previously for the three acetonides shows that no definite conclusions may be drawn from the available data. The positions of protons i and j in the acetonides would have to be determined by decoupling experiments. Even with this data the results would have to be interpreted with caution. The magnitude of the shift due to this long range effect is expected to be small and other factors related to distortion of the molecule or electrostatic influences might be comparable to the long range shielding effect.

2. Ord and cd data

Semi-synthetic neoallocyathin A_4 acetonide was examined by ord and cd. Both spectra showed a positive Cotton effect curve. The cd spectrum determined in methanol had a positive maximum at 346 nm with a dichroic absorption of $\Delta\varepsilon=+0.80$ while in dioxane the positive maximum was shifted to 354 nm with a dichroic absorption of +0.77. Both the nature of the Cotton effects and the shift of the cd maximum to higher wavelength in the less polar solvent are characteristics of the $n \rightarrow \pi^*$ transition in α,β-unsaturated ketones.

The acetonide prepared from natural neoallocyathin A_4 gave ord and cd curves similar to those from the synthetic material. The spectra showed positive Cotton curves and the cd had a positive maximum at 346 nm with a dichroic absorption of $\Delta\varepsilon=+0.57$.

Both the natural and synthetic samples also show two negative peaks in the cd spectrum at 300 and 270 nm in methanol and at 310 and 260 nm in dioxane. The origin of these peaks is not obvious since the α,β-unsaturated ketone is expected to give rise only to the positive maximum mentioned above in the region above 250 nm. The presence of identical impurities in both the natural and synthetic samples is unlikely unless the impurity arises from a common decomposition product.

It was also noted that the magnitude of these negative peaks was greater in the natural material than in the synthetic sample. However, after standing at room temperature for several weeks the synthetic samples showed an increase in these peaks so that its cd spectra more closely resembled that of the natural acetonide. The details of these spectra are presented in the experimental section.

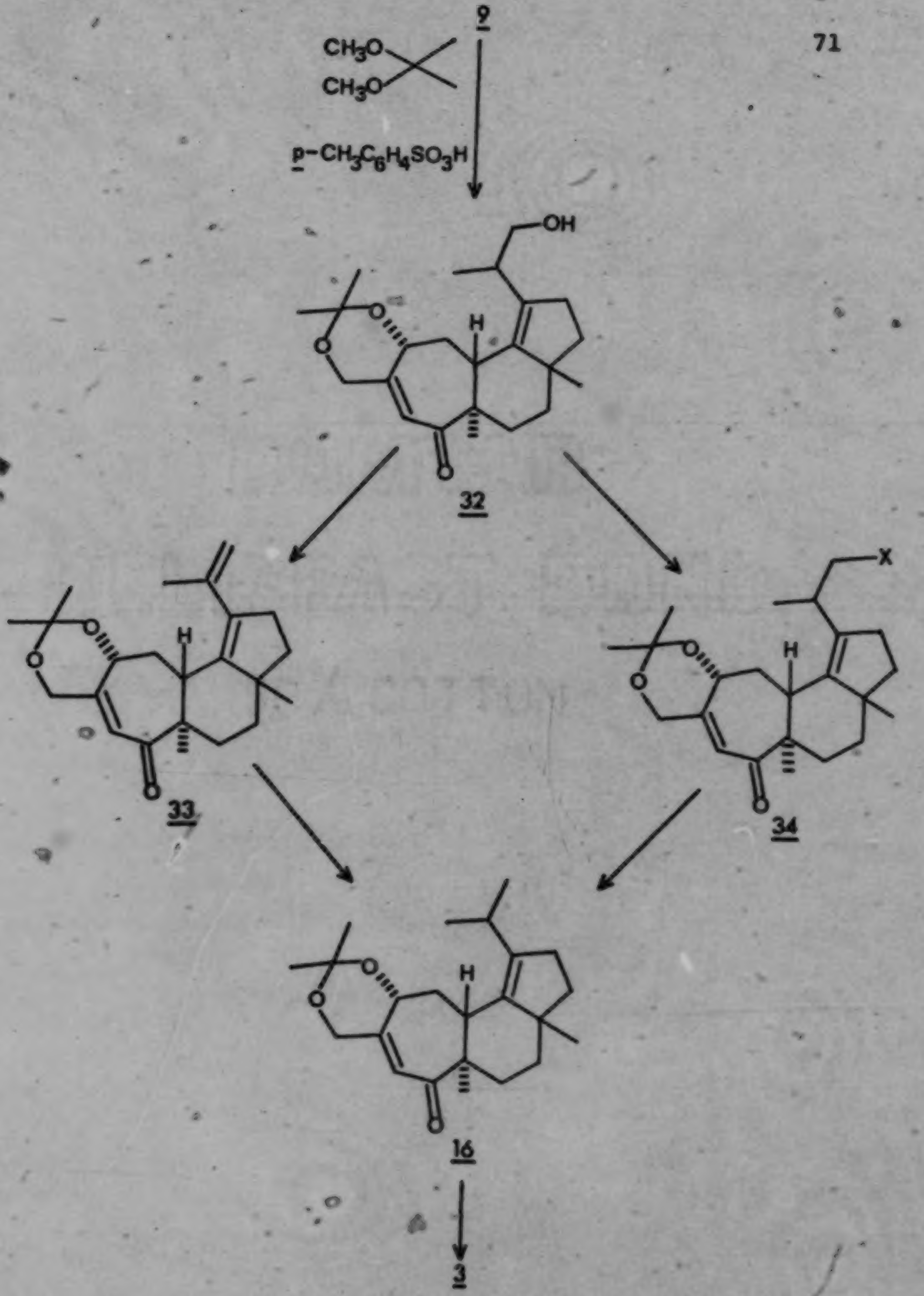
The uv spectrum was determined in methanol with the synthetic sample. Absorption maxima occur at 232 nm ($\varepsilon \sim 12{,}000$) and at 322 nm ($\varepsilon \sim 100$). This absorption corresponds to the $\pi \longrightarrow \pi^*$ and $n \longrightarrow \pi^*$ transitions expected for an α,β-unsaturated ketone. The cd maximum is expected to coincide roughly with the uv $n \longrightarrow \pi^*$ transition. Since the cd maximum occurs at 346 nm and the uv maximum occurs at 322 nm, this is obviously not the case. It is possible that the maximum expected in the uv at ~346 nm may have been concealed by the absorption at 322 nm, this latter peak representing some impurity in the sample. Alternately, the uv peak at 322 nm may be due to addition of the end absorption of the $\pi \longrightarrow \pi^*$ transition, while in the cd these bands are cleanly resolved. Further investigation would be necessary to clarify this apparent anomaly.

Toward the Structure Proof of Cyathin A_4

Cyathin A_4 was assigned structure 9 on the basis of spectroscopic evidence on the parent compound and several derivatives[9,10]. The tentative structure 9 differs from cyathin A_3 (3) only in that it contains an hydroxyl group at C-19 while cyathin A_3 contains a hydrogen at this position.

As a verification of the tentative structure 9 several reaction sequences were considered which would allow conversion of cyathin A_4 (9) into cyathin A_3 (3). Since the structure and absolute stereochemistry of cyathin A_3 are known the proposed structure 9 for cyathin A_4 would be verified.

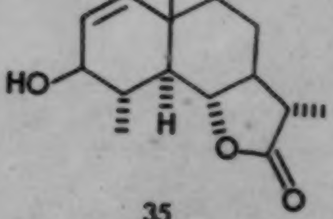
Scheme VI outlines two approaches to this conversion. Treatment of the acetonide 32 with a dehydrating agent would lead to the triene 33. Selective reduction of the exocyclic double bond followed by hydrolysis of the acid labile blocking group would lead to cyathin A_3 (3). Alternately, the acetonide 32 could be converted to compound 34 in which the hydroxyl group has been replaced by some functionality (eg., halogen) which could be replaced by hydrogen.



Scheme VI

The dehydration-reduction sequence was attempted starting from a sample of cyathin A_4 isolated (by L. Carstens) by column chromatography and purified by ptlc. The acetonide 32 was prepared and characterized by its mass, ir and nmr spectra as described in the experimental section. These spectra compared favourably with those obtained by other workers for this compound[9,10].

A procedure which involved heating of the alcohol in the presence of neutral alumina to which 1 or 2% pyridine has been added was considered for the dehydration step. This reagent was developed by von Rudloff[21] and has been used for the dehydration of monoterpene and sesquiterpene alcohols as well as other natural products and derivatives [22,23]. Corey and Hortmann used the reagent during the synthesis of dihydrocostunolide, converting the alcohol 35 to the diene 36[22].



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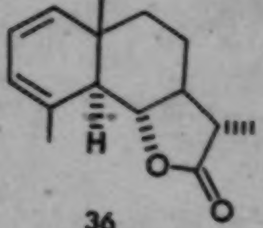

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The main advantage of this reagent over acidic reagents appears to be the inhibition of carbonium ion formation and hence suppression of isomerization of the double bonds. In this particular dehydration acidic reagents must also be avoided due to the presence of the acid labile blocking group in the acetonide 32.

After heating the catalyst and sample at 230° C for one hour the products were recovered by extraction of the reaction mixture with ether. The 30% of material recovered showed at least five components by tlc and no effort was made to further characterize the mixture. A second dehydration was attempted at a lower temperature but the mass spectrum of the product gave no indication that dehydration had taken place and the complex composition of the product discouraged further work with this method.

The second sequence illustrated in Scheme VI could be carried out by treatment of the acetonide 32 with phosphorus trihalide (eg., PBr_3) or a related reagent, followed by reaction of the halide with tri-n-butyltin hydride to give cyathin A_3 acetonide (16). Alternately reaction of the alcohol 32 with methyltriphenoxyphosphonium iodide followed by selective reduction with sodium cyanoborohydride in hexamethylphosphoric triamide would lead to the acetonide 16. This reagent for iodination was developed by Rydon and co-workers[24] and has found considerable use in alkyl halide formation[25]. The reducing agent is selective toward the primary iodo functionality in the presence of other reactive functionalities such as epoxides, ketones or aldehydes[26]. This reducing agent is therefore preferable to tri-*n*-butyltin hydride which is known to reduce the carbonyl function in aldehydes and ketones.

This correlation could not be attempted since we were unable to isolate more cyathin A_4. Crude samples isolated during and after the initial dehydration studies were found to lack cyathin A_4 despite its abundance in earlier growths. No further cyathin A_4 has been detected in the crude cyathin metabolites.

Cyathin C_5 - Structural Assignment from Data Supplied by Dr. A. D. Allbutt[10]

Allbutt succeeded in isolating a cyathin component named cyathin C_5. At the time of his work the structure remained unsolved. This compound can now be assigned a structure that is in agreement with data obtained for other cyathin components. The isolation and spectra discussed below for cyathin C_5 are obtained from reference 10.

A crude extract of C. helenae was separated by ptlc using a solvent system composed of benzene:1,4-dioxane:acetic acid, 100:25:1. A band at R_f 0.42 crystallized in ethyl ether-pentane. The crystalline material, melting point 225 - 226° C, constituted one third of the weight of this band at R_f 0.42. The ir spectrum (nujol) shows bands at 3400 and 3200 cm^{-1} (hydroxyl), 1695 and 1675 (carbonyl) and 1235 and 1220. The mass spectrum (figure 17) shows a probable parent ion at m/e 346. High resolution mass spectrometry gave an exact mass of m/e 346.1774 corresponding to $C_{20}H_{26}O_5$. Other prominent peaks occurred at m/e 300, 233, 91, 74, 59, 58, 45, 43, 41 and 31 (100%). The uv spectrum (MeOH) gave λ_{max} 215 nm ($\varepsilon \sim 11,000$) and λ_{max} 220 nm ($\varepsilon \sim 13,000$).

Figure 17. Mass spectrum of cyathin C_5.

Figure 18. Nuclear magnetic resonance spectrum (acetone-d_6) of cyathin C_5.

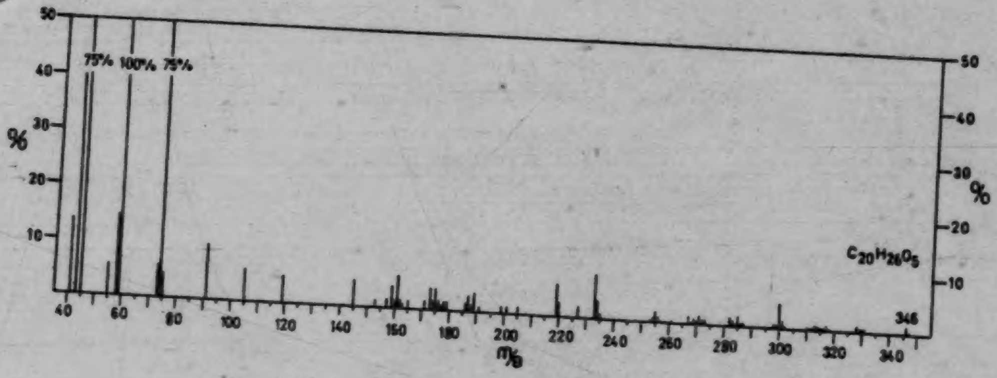


Figure 17

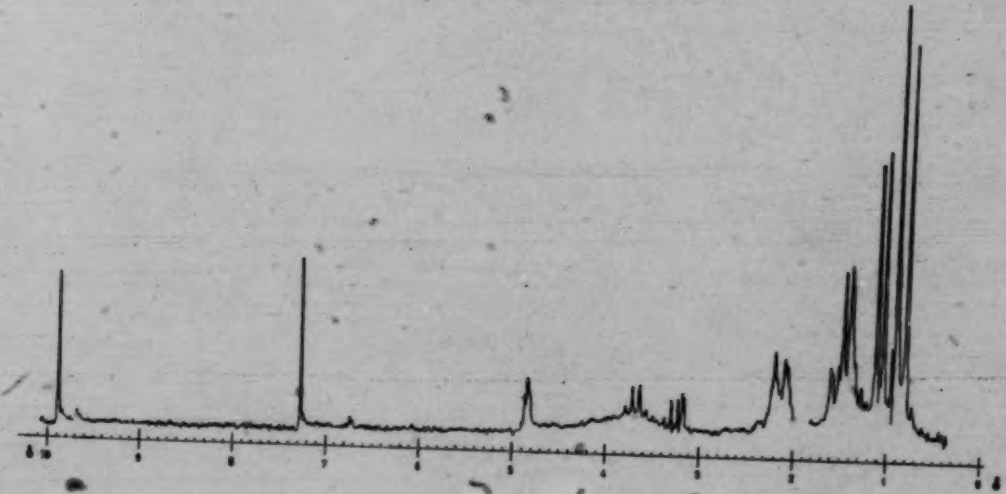


Figure 18

Analysis of the nmr spectrum (acetone-d_6) reproduced in figure 18 and Table 4 makes it possible to assign a tentative structure for cyathin C_5 especially when considered in conjunction with spectra for cyathin B_3 (5)[7,8]. The relevant signals from the nmr spectrum of cyathin B_3 are included in Table 4.

The three signals at lowest field in cyathin B_3 are due to features found in ring C. The signal at δ 9.91 was assigned to the aldehyde proton, its position being characteristic of an α,β-unsaturated aldehyde. The signal at δ 7.06 was assigned to the olefinic proton on C-13 and the signal at δ 5.08 to the C-11 proton. The low field position of this last signal is due to the proton being simultaneously allylic, α to an hydroxyl and vinylogously α to a carbonyl group. In the nmr spectrum of cyathin C_5 three signals correspond to the signals in cyathin B_3, both in approximate chemical shift (δ 9.89, 7.27 and 4.84) and in multiplicity (s, s, unresolved multiplet). This evidence indicates that cyathin C_5 contains a C ring equivalent to cyathin B_3.

The methyl region of the nmr spectrum of cyathin C_5 contains singlets at δ 0.92 and 0.81 and a doublet at δ 1.08 with J = 7Hz. The doublet at δ 1.08 is coupled with

Nmr data for cyathin C_5 (37)

Signal	Chemical shifts (δ) Cyathin C_5	Cyathin B_3 [(a)]	Multiplicity	Coupled with signals	Coupling constant (Hz)
b	9.89	9.91	s	-	-
e	7.27	7.06	s	-	-
h	4.84	5.08	u	i,j	?
x	3.69	-	q	m	7
m	1.08	-	d	x	7
n	0.92	1.08	s	-	-
o	0.81	0.94	s	-	-

The remaining signals appear as an unresolved multiplet between δ 2.40 and 1.20.

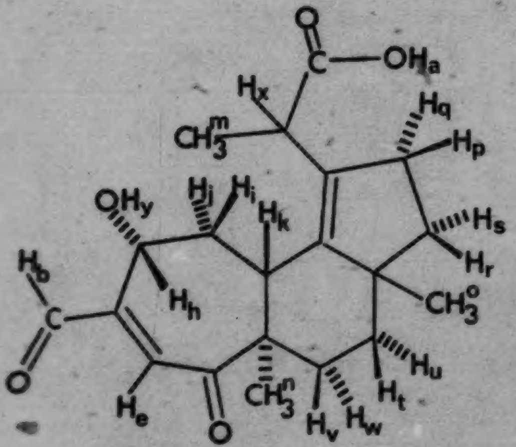
The absence of signals for the acidic proton Ha and the alcohol proton Hy may be explained by an exchange process involving these two protons.

(a) data from reference 6.

Table 4

a quartet at δ 3.69 with J = 7Hz. Spin-spin decoupling by irradiation of this latter signal led to collapse of the doublet centered at δ 1.08 to a singlet at the same position.

The nmr data discussed above is consistent with a normal cyathin skeleton containing a C ring equivalent to cyathin B_3 (5). The lack of further splitting of the quartet for a methine proton at δ 3.69 indicates that there are no protons α to this proton except those in a methyl group. The low field position of this signal suggests that the proton is influenced by one or more strongly deshielding groups. Remembering that our molecular formula requires the addition of two oxygens outside of the C ring it appears likely that cyathin C_5 is the carboxylic acid 37.



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The proton x would give rise to a quartet since this proton is coupled only to a methyl group. The deshielding by both the double bond and the acid functionality would account for the low field position of this signal (δ 3.69).

A signal for the acidic proton a was not observed in the nmr, perhaps due to failure to scan the spectrum above δ 10.0. The hydroxyl proton Hy may be concealed in the signals between δ 3.0 and 4.0. D_2O exchange did not resolve the situation since the signal for HOD obscured part of this region. It is also possible that the acidic proton and the hydroxyl proton may be exchanging and thus averaging in the δ 3.0 to δ 4.0 region.

Cyathin C_5 was isolated from crude cyathin on only one occasion. Later growths of C. helenae did not produce detectable quantities of this metabolite. Confirmation of the structure proposed above must therefore await further isolations of this compound from the crude mixture. Correlation with cyathin A_4 is also conceivable, provided that this compound becomes available again.

Biological Activity of Cyathin B_3-Cyathin C_3

As mentioned previously, mixed crystals of cyathin B_3-cyathin C_3 were readily obtained during the isolation procedure described. Through the cooperation of Dr. J. A. Weisbach, Smith, Kline and French Laboratories, Philadelphia, these have been screened for antibacterial and antifungal activity. The results of these tests are given in the Appendix. As may be noted, the mixed crystals showed interesting activity against Trichophyton mentagraphytes, a fungus related to those causing athlete's foot and other skin problems. Carstens had been unable to cleanly separate cyathin B_3 from cyathin C_3. During the course of this work it was found that ptlc on freshly prepared silica gel plates (see detailed experimental for a description of the method used) brought about separation of these two substances. It was of interest to see whether the biological activity mainly resides with one or the other component and accordingly the individual components were submitted for testing. As shown in the Appendix, cyathin B_3 appears to be more active than cyathin C_3, but the difference is small and the individual components appear less active than the mixture.

Metabolites of Crucibulum Vulgare

Initial screening of species of bird's nest fungi indicated that a crude extract of Crucibulum vulgare had antibacterial properties. This result was at variance with a previous report on the absence of antibacterial action with this species[3]. This fungus grew well on surface culture under the same conditions described for C. helenae .

Some variation in the media was attempted. At one point a 10 fold increase in concentration of Brodie medium nutrients in the broth was used but this did not result in an increase in metabolite production. The use of Czapek-Dox medium was examined. This medium, especially suited for the laboratory cultivation of fungi (reference 15, page 16), gave a poor production of metabolites. The best results were obtained with normal concentration of Brodie medium.

Despite a rapid growth of mycelium on surface culture the yield of metabolites was low. The crude material isolated from 15 separate growths varied from 35 - 84 mg from each liter of media extracted (compare with C. helenae which yielded 150 - 600 mg per liter).

Preliminary investigation of the crude mixture by column chromatography and ptlc revealed a complex mixture. After unsuccessful attempts at isolation of pure components from the mixture it was decided to abandon the project until methods which allowed the production of larger amounts of metabolites could be developed. During the investigation of the crude materials some information was obtained indicating the nature of metabolites obtained from C. vulgare.

The crude material revealed a consistent pattern on tlc. With solvent system A the developed plate showed three prominent spots at R_f 0.66, 0.59 and 0.53. These areas were visible when a phosphor impregnated plate was viewed with uv light and they charred a brown or grey colour after treatment with sulfuric acid and heat. These three areas appeared to be most suitable for initial investigation.

Developed and sprayed tlc plates also revealed materials at lower and higher R_f positions. The materials at higher R_f varied for each crude and were present in minute amounts. Materials at lower R_f were numerous and usually appeared as a streak of material rather than isolated areas. Ptlc revealed that a large portion of material remained at the origin in solvent system A; often as much as 50% of the weight of applied sample could be recovered by extraction of the silica below R_f 0.10 on the developed plate. Trials with a series of other solvent systems did not improve the separation.

Attempts to isolate the three materials between R_f 0.66 and 0.53 were unsuccessful although a degree of refinement was obtained by combined column chromatography and ptlc. The materials in this R_f range showed mass spectra with peaks above m/e 400.

The least polar uv active fraction at R_f 0.59 was examined by various spectral means. High resolution mass spectrometry indicated a peak at m/e 440 as the probable parent ion with a molecular formula $C_{30}H_{48}O_2$. Additional peaks were present above and below m/e 440 which could not be readily derived from this parent ion. This indicated that the sample was not of high purity.

The ir spectrum showed absorption at 1735 and 1645 cm^{-1} and a sharp absorption at 1585 cm^{-1}. Some samples showed a broad weak hydroxyl absorption but the inconsistency of this absorption in different samples suggested that it was not due to the main component of the fraction.

A series of signals in the nmr from δ 6.0 to 6.6 indicated the presence of olefinic protons although the small size of these signals relative to the rest of the spectrum suggests that they are due to a minor component in the sample.

The most polar fraction examined, R_f (A) 0.53 appeared to contain at least three components. Acetylation of this fraction after purification by ptlc gave a material the tlc of which showed three spots. The ir of the most polar of the acetylated components showed a strong absorption at 1715 cm^{-1} and a series of peaks from 3000 to 2400 cm^{-1} suggesting that one or more of the components of the mixture was an acid. Other than the mass spectrum, which indicated peaks at m/e 428 and 426 as possible parent ions, no further characterization was carried out on this fraction.

The fraction at R_f (A) 0.59 gave only traces of material after ptlc of crude samples. The spectra of this fraction were weak and poorly defined.

The exact mass determination and the observation of m/e values in excess of 400 for the three fractions examined, indicated that the compounds were perhaps triterpenoid or steroidal in nature. The failure to detect diterpenoid metabolites with some relationship to the cyathins from C. helenae was another factor which lead to the discontinuation of this project.

III GENERAL EXPERIMENTAL

1. Growth of fungi and collection of crude fungal metabolites.

The procedures described briefly in this section have been described in more detail by others[2,4]. Fruiting bodies of the bird's nest fungi were sectioned after sterilization of their surface. Incubation of the sections on a sterile nutrient agar encouraged a growth of monosporus mycelia to cover the agar surface. Blocks of agar containing mycelial growth were transferred to fresh nutrient agar and this growth and transfer procedure was repeated until an homogeneous growth of mycelium was obtained. The mycelium was stored on petri plates or slant tubes of solid medium kept at approximately 0° C. The transfer to large scale growths on liquid media involved the removal of "plugs" of the mycelium from a petri plate. These plugs were transferred to 500 ml Erlenmeyer flasks containing 250 ml of sterile medium and the growth was allowed to proceed for three to four weeks. The content of these flasks was then ground up in a sterile blender and pipetted into a number of 2 l Fernbach flasks containing 500 ml of sterile medium.

After 25 to 35 days growth the dark brown medium was poured from the flasks leaving behind the solid pad of mycelium. The mycelium could then be reflooded with fresh sterile medium. This reflooding technique was repeated until the flasks became contaminated or until the bulk of mycelium in the flask made the procedure impractical.

The culture broth was twice extracted with half volumes of ethyl acetate. The organic layer was dried with anhydrous sodium sulfate and the solvent was removed on a rotary evaporator. On some occasions the solution was filtered before evaporation of the last 50 to 100 ml of solvent to remove quantities of insoluble material.

2. Solvents and adsorbents

All solvents used in this work were distilled prior to use except anhydrous ethyl ether which was used directly from the original container. The small quantities of involatile materials found in most reagent grade solvents were intolerable in the small scale separations necessary in this work and prompted the distillations. The commonly used adsorbents were silicic acid (Mallinckrodt, 100 mesh) and silica gel G (E. Merck). These adsorbents were assumed to be of high purity and no further refinement of these solids was attempted.

3. Column chromatography

A quartz column 4.0 cm in diameter and 50 cm in length was used for separation of crude metabolites. Silicic acid (Mallinckrodt, 100 mesh) was the only adsorbent used for column chromatography. Addition of 1% inorganic phosphor (General Electric, type 118-2-7) to the adsorbent allowed the progress of the separation to be observed by irradiation of the column with uv light.

After preparation of the column the adsorbent was allowed to settle for 12 to 16 hours before use. The sample was usually applied as a concentrated solution in a small quantity of the eluting solvent. Fractions of 50 to 125 ml were collected manually. Alternately 5 to 20 ml fractions were collected by an automatic fraction collector (Isco, Golden Retriever).

4. Thin-layer chromatography

Aqueous slurries of silica gel G (E. Merck) were spread into thin layers on glass plates of various sizes using DESAGA equipment in a procedure described by Stahl[27]. The resulting thin layers of adsorbent were air dried for several hours and then dried in an oven at 110° to 115° C for several hours to activate the adsorbent. Efforts were made to store the plates away from atmosphere moisture although it was found more practical to reactivate the plates prior to use by reheating to 110° C. Alternately the plates were used in the ambient state of activity. The tlc behaviour of materials on freshly activated plates was not strikingly different from that on older plates.

The same basic procedure was used for the preparation of $AgNO_3$ impregnated plates. The $AgNO_3$ was dissolved in water and this solution was added to the silica gel. After preparation the plates were protected from light and dust as much as possible. Efforts were made to use the plates on the same day as their preparation since they deteriorated rapidly.

Preparative work was carried out on plates 20 cm in height and varying in length from 10 to 100 cm with an adsorbent layer of approximately 0.5 mm. Analytical work was carried out on plates 20 cm in height with lengths from 5 to 20 cm. The analytical plates were scored into sections prior to use as illustrated in figure 1. This procedure helps to prevent the horizontal spreading of the sample as it moves up the plate. Smaller plates prepared by coating microscope slides with adsorbent were used to follow the course of reactions and to analyze column fractions.

Sample application for analytical work involved the use of micropipets prepared by heating open ended capillary tubes (1.6 mm x 100 mm) and drawing them out to a fine thread of approximately 0.1 mm in diameter. Samples in solution were drawn up into these pipets by capillary action and delivered to the prepared plates by touching the adsorbent layer at a position about 2 cm from the bottom edge.

In preparative work the applicator was prepared by drawing out the end of a disposable pipet to a fine thread. The sample in concentrated solution was then drawn into a pipet with a rubber bulb and applied to the plate by drawing the tip of the glass thread across the plate while applying pressure to the bulb. Samples of 200 mg could be separated on the 20 x 100 cm plates using this procedure.

Plates were developed in enclosed tanks until the solvent front just reached the top edge of the silica. This required 45 minutes to one hour for the plates 20 cm high and 10 minutes for the microscope slides.

The following solvent systems were employed at various times for development of the tlc plates:

A	benzene:acetone:acetic acid	75:25:1
B	acetone:skelly B	20:80
C	acetone:skelly B	30:70
D	acetone:skelly B	50:50
E	acetone:skelly B	70:30
F	toluene:acetone:acetic acid	72:25:1

Components of the mixture were located with a H_2SO_4 spray or by uv irradiation of phosphor impregnated plates as discussed previously. A third method was employed with $AgNO_3$ impregnated plates. The plates were sprayed lightly with a 0.2% solution of 2',7'-dichlorofluorescein in ethanol followed by viewing under long wave uv light at 325 nm.

The position of a band or spot on a plate is recorded as its R_f value. The use of argentated chromatography is indicated by placing "$AgNO_3$" in brackets along with the letter indiating the solvent system in use. For example R_f (B, 10% $AgNO_3$)0.23, indicates that in solvent system B a compound showed an R_f value of 0.23 when run on a plate impregnated with 10% $AgNO_3$.

After removal of the bands from preparative plates the samples were washed from the silica with various solvents. With $AgNO_3$ impregnated plates ethyl ether was used exclusively since other commonly used solvents were found to remove quantities of $AgNO_3$. The silica was filtered from the solvent using a fritted glass funnel (porosity E, 4 to 8μ).

After chromatographic purification and removal of solvent, samples were submitted to the various spectroscopic laboratories for the determination of their spectra.

Spectral Determination

Mass spectra were recorded on an A.E.I. Model MS-2 or an A.E.I. Model MS-9 mass spectrometer. The latter instrument was used to determine molecular weights by high resolution mass measurement. The small size of isolated samples did not allow confirmation of molecular formulas by microanalysis. All mass spectra are recorded in this thesis as a percent of the base peak.

Nmr spectra were recorded on a Varian Associates HR-100 spectrometer with tetramethylsilane as an internal reference. Spectra of small samples (0.5 - 5 mg) were recorded on a Varian Associates HA-100 15" instrument by Pulse Fourier Transform using the Digilab Data System and pulser. The pattern of signals is denoted by: s=singlet, d=doublet, t=triplet, q=quartet, m=unresolved multiplet, b=broad, u=unresolved band.

Ir spectra were recorded on a Perkin-Elmer Model 421 dual grating spectrophotometer or a Unicam SP 1000 infrared spectrophotometer.

Uv spectra were recorded on a Cary Recording Spectrophotometer, Model 15.

Cd and ord spectra were recorded on a Durrum-Jasco Recording Spectropolarimeter.

Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

IV DETAILED EXPERIMENTAL

Growth and Isolation of Crude Cyathin

On the 16th of October 1973, 10 liters of Brodie medium was prepared. This medium contains the following nutrients per liter: glycerine, 6 ml; peptone, 0.2 g; dl-asparagine, 0.2 g; yeast extract, 2.0 g; $MgSO_4$ (anhydrous), 0.24 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; KH_2PO_4, 0.5 g; $Fe_2(SO_4)_3$, trace; maltose, 5.0 g; dextrose, 2.0 g. The medium was placed in 18 2 l Fernbach flasks and the stoppered flasks were autoclaved at 127° C and 15 lb pressure for 15 minutes. When the flasks had cooled to room temperature they were innoculated with mycelia from a growth labeled "J" prepared by L. Carstens. This sample growing on surface culture was ground to a fine suspension and pipetted into the sterile Brodie medium

On the 19th of November after a growth period of 34 days the medium was removed from the flasks leaving the mycelium behind. The mycelium was reflooded with 9 liters of sterilized Brodie medium. The 8.5 liters of brown liquid removed from the flasks was extracted with ethyl acetate. The extract yielded 2.59 g of brown foam after evaporation of solvent.

Isolation of Neoallocyathin A_4 Acetonide

The isolation procedure is summarized in scheme IV and is detailed here by a specific example from the author's work.

1. Column chromatography

Crude material (1.01 g) was chromatographed using a 4.0 x 50 cm quartz column containing 68 g of silicic acid (Mallinckrodt, 100 mesh) and 0.68 g of inorganic phosphor. The sample was applied to the column as a concentrated solution and the elution was carried out with 1.5% methanol in redistilled reagent chloroform. Fractions were examined by tlc. Eighteen 125 ml fractions were collected and the compounds of interest were observed by tlc to be mainly in fractions 9, 10 and 11. These fractions were combined to give 214.9 mg of material after removal of the solvent.

2. Tlc on silica gel

The 214.9 mg of material from above was dissolved in chloroform and spread on a 20 x 100 cm plate containing a 0.5 mm layer of silica gel G (E. Merck) containing 5%

added inorganic phosphor. The plate was developed with solvent system E and one main band, R_f (E)0.66, was removed. A clear oil (56.8 mg) was eluted from this band. The mass spectrum of the material is similar to that reproduced in figure 2. Since it was obvious that the material was a mixture, no more specific observations of the nature of this material were undertaken before further refinement.

3. Tlc on argentated silica gel

The 56.8 mg of clear oil obtained from preparative tlc was spread on one 20 x 20 cm plate containing a 0.5 mm layer of silica gel G (E, Merck) impregnated with 10% $AgNO_3$. The plate was developed with solvent system E. After drying the plate was sprayed lightly with 2',7'-dichlorofluorescein and observed under long wave uv light. Two bands were observed. The upper band, R_f (E, 10% $AgNO_3$)0.59, gave 14.9 mg of clear oil when eluted with ethyl ether. The mass spectrum of this material is reproduced in figure 5.

Exact mass on the peak at m/e 334 indicated the molecular formula $C_{20}H_{30}O_4$ and that of the fragment at m/e 319 indicated the formula $C_{19}H_{27}O_4$. Exact mass on m/e 318 and m/e 303 indicated the molecular formulas $C_{20}H_{30}O_3$ and

$C_{19}H_{27}O_3$ respectively.

The lower band, R_f (E, 10% $AgNO_3$)0.50, gave 18.0 mg of yellow oil. The tlc behaviour of this material was identical with that of allocyathin B_3 (4) on both argentated silica gel, R_f (E, 10% $AgNO_3$)0.50, and silica gel, R_f (E)0.66, R_f (A)0.23. The mass spectrum of this material, reproduced in figure 3, compares favourably with that of allocyathin B_3 (figure 4). Later characterization of this material as its acetonide and diacetyl derivatives served to confirm its identity with allocyathin B_3 (4).

4. Acetonide formation

The 14.9 mg of clear oil from above was dissolved in 2 ml of 2,2-dimethoxypropane and a small crystal (~1 mg) of *p*-toluenesulfonic acid was added. The resulting solution was stirred at room temperature for 30 minutes. The volume of the solvent was reduced using a rotary evaporator and the remaining solution was spread directly onto a 20 x 20 cm plate containing silica gel G (E. Merck). Two materials were noted on the plate after development. A small quantity of material was recovered from a band at R_f (A)0.46. The majority of material was located at a slightly higher position, R_f (A)0.57. The 8.8 mg of material isolated from this band appeared to be a mixture of acetonides. Mass spectrum: m/e 374(19), 359(20), 358(28), 345(16), 343(17), 315(7), 285(27), 91(17), 55(72), 45(35), 43(100), 41(40), 39(15); ir ($CHCl_3$): 3520 and 3600 - 3000 cm^{-1} (weak hydroxyl), 1650 cm^{-1} (ketone). The weak hydroxyl absorption was not present in later infrared spectra of this sample and is therefore assumed to be due to a volatile hydroxylic impurity.

5. Separation of acetonides

The 8.8 mg of material recovered from ptlc with solvent system A was separated by spreading on 2 20 x 20 cm plates containing silica gel G (E. Merck) and developing with solvent system B. The upper band, R_f (B)0.51, gave 5.8 mg of clear oil after removal from the silica. Tlc on this material showed a single spot which charred brown on treatment with H_2SO_4 spray followed by heating. The lower band, R_f (B)0.49, gave 2.0 mg of clear oil which developed as a violet spot after spraying with sulfuric acid. The mass spectrum indicated that the 2.0 mg of material was composed almost entirely of neoallocyathin A_4 acetonide. Cd (c, 0.0031, MeOH):$\Delta\varepsilon_{346}$ + 0.57, $\Delta\varepsilon_{300}$ - 0.45, $\Delta\varepsilon_{268}$ - 0.10; ord (c, 0.0031, MeOH):

Φ_{374} + 2439°, Φ_{323} − 5203°, Φ_{346} − 1951°, shoulder below 300 nm. The mass ir and nmr spectra are reproduced in chapter 2.

Acetylation of Fractions Obtained from Preparative Tlc on Argentated Silica Gel

The high R_f mixture obtained after argentated chromatography (18.4 mg) was dissolved in 5 ml of CH_2Cl_2. To this solution was added 20 drops of pyridine and 10 drops of acetic anhydride. The sample was left at room temperature overnight. One major band was removed after ptlc. Elution with acetone yielded an oil (9.8 mg); R_f (A)0.65. The ir and mass spectra are reproduced in figures 7 and 8 respectively. Nmr($CDCl_3$); δ 6.15 (1 H, u, olefinic), 5.40 (2 H, u, $-C\underline{H}_2-OCOCH_3$), 4.75 (1 H, u, $-C\underline{H}-OCOCH_3$) 2.10 and 2.18 (3 H, s, acetyl methyl groups). These signals and the methyl region signals are similar to those in the spectrum of O,O-diacetylcyathin A_3 reproduced elsewhere (reference 6, pages 50 and 51). The main component of the mixture appears to be the diacetyl derivative of cyathin A_3.

The low R_f mixture obtained after argentated chromatography (20.1 mg) was treated with 0.25 ml of pyridine and 5 drops of acetic anhydride. The reaction was judged (by tlc) complete after 30 minutes. The product was purified by ptlc and characterized as O,O-diacetylallocyathin B_3. R_f (A)0.65; mass spectrum: m/e 400(23), 357(13), 297(12), 237(18), 209(17), 202(15), 201(48), 187(15), 173(16), 159(20), 145(16), 135(15), 134(19), 133(15), 119(35), 117(31), 105(17), 91(18), 43(100); nmr ($CDCl_3$): δ 6.29 (2 H, s, olefinic), 6.12 (1 H, s, olefinic), 5.37 (1 H, dd, J-6.5Hz, 6.5Hz, C-11 proton), 4.68 (2 H, u, $-C\underline{H}_2-OCOCH_3$), 2.08 and 2.00 (3 H, s, acetyl methyl groups), 1.08 and 1.06 (3 H, d, J=7Hz, isopropyl methyls), 1.01 and 0.98 (3 H, s, quaternary methyls), remainder of spectra unresolved. This data agrees with that previously published for O,O-diacetylallocyathin B_3 (see reference 6, pages 78 and 79).

Preparation of Neoallocyathin A_4 Acetonide from Cyathin A_3

Cyathin A_3 ($\underline{3}$, 17.6 mg) was obtained from crystalline samples prepared by H. Taube. This was dissolved in 5 ml of 2,2-dimethoxypropane to which a small crystal of p-toluenesulfonic acid had been added. The reaction was complete after 15 minutes as judged by tlc. The volume of solvent was reduced to 0.5 ml on the rotary evaporator and this solution was spread on a 20 x 20 cm preparative tlc plate and eluted with solvent system C. The major band yielded 13.5 mg of partially crystalline material. This material was identified as cyathin A_3 acetonide: R_f (B)0.51; mass spectrum: m/e 359(30), 358(100), 343(49), 315(25), 300(22), 286(20), 285(88), 257(52), 203(40), 189(37), 149(30), 123(32), 121(31), 119(31), 107(30), 105(37), 96(50), 91(31), 55(45), 43(55), 41(51); ir ($CHCl_3$): 2950, 2920 and 2860 cm^{-1} (aliphatic C-H), 1660 (α,β-unsaturated ketone), 1440, 1375, 1365, 1135, 1080, 1065, 1010; nmr ($CDCl_3$): reproduced in chapter 2.

Cyathin A_3 acetonide (13.5 mg) was dissolved in 5 ml of chloroform. Chloroform (6.5 ml) containing 1 mg/ml of m-chloroperbenzoic acid was added and the solution was stirred at room temperature for five minutes. The chloroform solution was washed twice with 10 ml of 5% $NaHCO_3$, twice with water and once with saturated brine. The chloroform was dried with sodium sulfate and evaporated under reduced pressure to yield 13.6 mg of clear oil. This sample was crystallized from a saturated ether-pentane solution and recrystallized in the same system to give white crystals; melting point 144.5 - 146.0° C; uv (MeOH):

λ_{max} 232 nm (ε 12,400), 322 nm (ε 110); cd (c, 0.0039; MeOH): $\Delta\varepsilon_{346}$ + 0.80, $\Delta\varepsilon_{300}$ - 0.18, $\Delta\varepsilon_{270}$ - 0.23; (c, 0.0075; dioxane): $\Delta\varepsilon_{354}$ + 0.77, $\Delta\varepsilon_{310}$ - 0.41, $\Delta\varepsilon_{260}$ - 0.43; ord (c, 0.0039; MeOH): Φ_{368} + 1554°, Φ_{325} - 6190°, Φ_{345} - 2320°, shoulder below 300 nm. This material was identified as neoallocyathin A_4 acetonide as described in chapter 2.

Formation of Allocyathin B_3 Acetonide

A light yellow foam (117.4 mg) rich in allocyathin B_3 was purified by preparative tlc on 10% $AgNO_3$ impregnated silica gel plates. The major band, R_f (E, 10% $AgNO_3$) 0.50, gave 31.1 mg of clear oil after elution from the silica with ethyl ether. This material appeared to be mainly allocyathin B_3: mass spectrum: m/e 332(7), 317(13), 316(35), 301(17), 203(14), 202(12), 201(19), 189(29), 187(24), 159(21), 150(24), 135(24), 133(20), 119(38), 107(28), 105(24), 91(27), 73(27), 61(25), 55(26), 43(100); this compares closely with figure 5 for allocyathin B_3 except for the peaks at m/e 332 and 317 and the base peak.

Treatment of this sample with 2,2-dimethoxypropane (containing a trace of p-toluenesulfonic acid) for four hours at room temperature followed by ptlc on silica gel G gave 5.7 mg of allocyathin B_3 acetonide: R_f (C)0.59; mass spectrum: m/e 357(26), 356(100), 341(6), 255(20), 135(35), 134(71), 119(70), 105(21), 91(27), 67(20), 55(25), 43(70), 41(45), 39(22); nmr ($CDCl_3$); reproduced in chapter 2.

Formation of Cyathin A_4 Acetonide

A chromatographic fraction (200 mg) rich in cyathin A_4 yielded 59.5 mg of light brown foam as the major fraction after ptlc with solvent system E. This material appeared to be mainly cyathin A_4: R_f (A)0.11; R_f (E)0.61; mass spectrum: m/e 334(3), 319(6), 316(12), 298(15), 289(18), 275(20), 203(34), 191(22), 190(28), 189(60), 187(25), 177(25), 176(22), 175(100), 173(20), 163(26), 161(36), 169(22), 159(22), 147(22), 145(18), 121(22), 28(55).

Cyathin A_4 (9, 13 mg) was dissolved in 3 ml of 2,2-dimethoxypropane containing a trace of p-toluenesulfonic acid. The reaction mixture was left overnight after which time tlc indicated complete transformation to a single product; R_f (A) 0.50. The volume of solvent was reduced with a stream of nitrogen and the concentrated solution spread on a 20 x 20 cm ptlc plate and developed with solvent system A. The 7.4 mg of material recovered from the main band was characterized as cyathin A_4 acetonide; mass spectrum: m/e 374(26), 359(22), 345(11), 316(18), 301(25), 286(30), 285(34), 161(43), 159(25), 145(29), 135(28), 133(32), 121(73), 119(47), 109(36), 107(35), 105(55), 96(42), 95(25), 93(38), 91(55), 81(35), 79(38), 77(28), 69(35), 67(35), 55(62), 43(100), 41(72); ir ($CHCl_3$): 3520 cm^{-1} (hydroxyl), 1735, 1715, 1650 (acetyl carbonyls and α,β-unsaturated ketone), 1470, 1380; nmr ($CDCl_3$): δ 5.70 (1 H, u, olefinic), 4.30 (2 H, u, characteristic of C-15 methylene protons in acetonides of other cyathins), 3.48 (2 H, d, J=7Hz, $-CH-C\underline{H}_2-OH$), 3.05 (1 H, m, $-C\underline{H}-CH_2-OH$), 1.44 and 1.38 (3 H, s, acetonide methyl groups), 1.08 and 1.07 (3 H, s, quaternary methyl groups), 0.96 (3 H, d, J=7Hz, isopropyl methyl group).

Attempted Dehydration of Cyathin A_4 Acetonide

Cyathin A_4 acetonide (7.4 mg) was placed in a 5 mm diameter glass tube and covered with a layer of aluminia catalyst. The catalyst was prepared by thoroughly mixing 1 ml of pyridine with 50 g of alumina (Woelm-neutral aluminia-grade 1). The tube was heated at 230° C for one hour, the contents were extracted with ethyl ether and the solvent was removed. The residue was 2.5 mg of brown film which contained at least five components as indicated by tlc. This material was not further characterized. A second attempt at dehydration using 8 mg of cyathin A_4 acetonide and the same procedure as above but with the temperature at 154° C, was not successful. Tlc showed there was no major component in the mixture of products and the mass spectrum gave no evidence of dehydration products. This sample was not further characterized.

Separation of Cyathin B_3 and Cyathin C_3

Crude "cyathin" (1 g) was spread on four 20 x 100 cm preparative tlc plates. The plates had been heated at 110° C overnight and the sample was applied and development begun (in solvent system C) while the plates were still warm. Two bands were removed from the region of the plates in which cyathin B_3 and cyathin C_3 usually appear as a single band. Preliminary evidence indicates that the two components are cyathin B_3 (5) and cyathin C_3 (6); R_f (C)0.64; mass spectrum: m/e 316(15), 301(16), 298(6), 204(12), 203(12), 189(35), 161(16), 119(18), 105(21), 93(16), 91(25), 79(15), 77(15), 69(18), 55(35), 53(15), 43(30), 41(45), 28(100). R_f (C)0.57; mass spectrum: m/e 316(5), 314(27), 299(8), 281(6), 202(23), 201(45), 187(39), 159(25), 149(21), 145(22), 119(35), 105(36), 91(32), 83(26), 71(20), 69(20), 57(32), 55(43), 53(22), 45(23), 43(95), 41(100). These samples were submitted for biological testing and the results appear in the appendix.

Separation of Neoallocyathin A_4 from Cyathin A_3

Late in this investigation preliminary evidence was obtained suggesting that these two components could be separated by ptlc. A sample containing a trace of neoallocyathin A_4 in cyathin A_3 was spotted on a silica gel G plate. Multiple elution in solvent system C gave slight separation of the two components. The major material corresponded to cyathin A_3 while a trace of neoallocyathin A_4 appeared at slightly lower R_f. After five elutions the materials had resolved into two distinct spots: R_f (C)0.49 (corresponding to authentic cyathin A_3) and R_f (C)0.44 (corresponding to neoallocyathin A_4). This method of separation has not been examined further.

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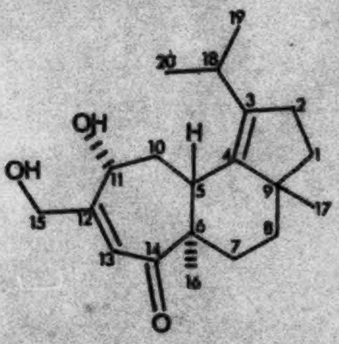
APPENDIX

The tables presented below represent a summary of the biological testing carried out by the Smith, Kline and French Laboratories, Philadelphia. The numbered blocks indicate the minimum inhibitory concentration (MIC) of the test samples against a variety of organisms. Both mixed crystals of cyathin B_3 and cyathin C_3 and the individual compounds were tested.

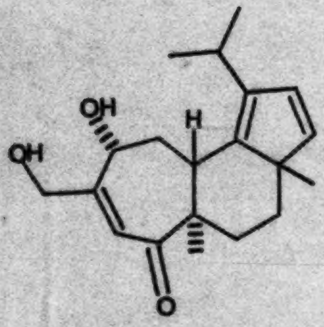
		PENASSAY SEED AGAR (pH 7.0)										MIC (µg/ml)				
CODE	SK&F NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		Staph. aureus HH 127	Staph. aureus SA 910	Strep. faecalis HH 34358	Mycobacterium phlei 1228	Proteus mirabilis PM 444	E. coli SKF 12140	Kleb. pneumoniae SKF 4200	Salmonella gallinarum ATCC 9184	Pseudomonas aeruginosa HH 63	Serratia marcescens ATCC 13880	Proteus morgani P-179	Providencia sp. PR-276	Enterobacter cloacae HH 31254	Candida albicans BC 759	Trichophyton mentagrophytes BC 1258
Cyathin B_3 JRM 334-1	75395	4	4	8	31	250	>250	>250	>250	>250	>250	63	>250	>250	125	4
Cyathin C_3 JRM 334-2	75396	4	8	16	31	>250	>250	250	>250	>250	>250	>250	>250	>250	125	8
Gentamicin		1.6	3.1	12.5	1.6	3.1	1.6	0.8	6.3	0.8	1.6	1.6	12.5	1.6	>200	200
Erythromycin		0.8	>200	>200	0.4	>200	50	50	>200	>200	200	100	>200	200	>200	>200
Amphotericin B		>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	0.2	1.6
Griseofulvin		>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	0.1

CODE	SK&F NO.	PENASSAY SEED AGAR (pH 7.0)								MIC (µg/ml)						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		Staph. aureus HH 127	Staph. aureus SA 910	Strep. faecalis HH 34358	Mycobacterium phlei 1228	Proteus mirabilis PM 444	E. coli SKF 12140	Kleb. pneumoniae SKF 4200	Salmonella gallinarum ATCC 9184	Pseudomonas aeruginosa HH 63	Serratia marcescens ATCC 13880	Proteus morgani P-179	Providencia sp. PR-276	Enterobacter cloacae HH 31254	Candida albicans BC 759	Trichophyton mentagrophytes BC 1258
JRM-1	74227	2	2	4	16	1000	>1000	63	63	>1000	>1000	63	63	>1000	63	1
Gentamicin		1.6	6.3	25	1.6	6.3	1.6	0.8	6.3	1.6	6.3	1.6	12.5	1.6	>200	>200
Erythromycin		0.4	>200	>200	0.2	>200	50	100	200	200	100	50	200	100	>200	>200
Amphotericin-B		>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	0.4	0.8



3

cyathin A_3



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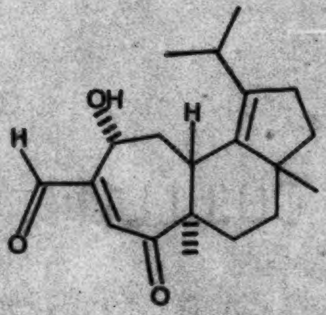
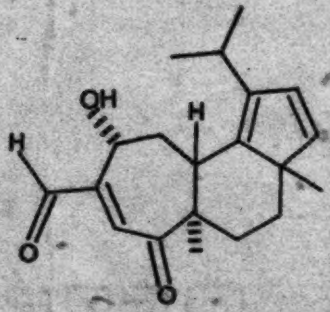
allocyathin B_3



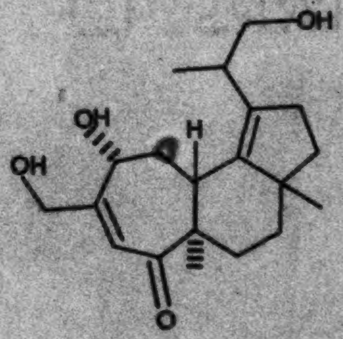
5

cyathin B_3



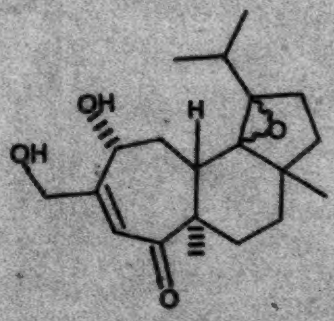
6

cyathin C_3



9

cyathin A_4



24

neoallocyathin A_4